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RESEARCH AND PREPARATION OF AN EQUINE HEPTAVALENT BOTULINAL ANTITOXIN

FINAL REPORT

Richard M. Condie

February 1989

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-82-C-2119

University of Minnesota
Minneapolis, Minnesota 55455

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90 03 15 067

AD-B141 387

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified			1b. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution authorized to U.S. Government agencies only; proprietary information April 1, 1982. <i>ATTN: SGRD-RMI-5</i>		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE			5. MONITORING ORGANIZATION REPORT NUMBER(S)		
4. PERFORMING ORGANIZATION REPORT NUMBER(S)					
6a. NAME OF PERFORMING ORGANIZATION University of Minnesota		6b. OFFICE SYMBOL (if applicable)		7a. NAME OF MONITORING ORGANIZATION	
6c. ADDRESS (City, State, and ZIP Code) Mayo Memorial Building Minneapolis, Minnesota 55455				7b. ADDRESS (City, State, and ZIP Code)	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command		8b. OFFICE SYMBOL (if applicable)		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-82-C-2119	
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012		10. SOURCE OF FUNDING NUMBERS			
		PROGRAM ELEMENT NO. 62770A		PROJECT NO. 3MI-62770A871	TASK NO. BA
				WORK UNIT ACCESSION N 049	
11. TITLE (Include Security Classification) RESEARCH AND PREPARATION OF AN EQUINE HEPTAVALENT BOTULINAL ANTITOXIN					
12. PERSONAL AUTHOR(S) Richard M. Condie, Ph.D.					
13a. TYPE OF REPORT Final Report		13b. TIME COVERED FROM 4/1/82 TO 7/31/83		14. DATE OF REPORT (Year, Month, Day) 1989 February	
15. PAGE COUNT 95					
16. SUPPLEMENTARY NOTATION					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP			
06	13		RA I; new heptavalent F(ab') ₂ botulinal antitoxin equine toxin; immunology; antitoxin; neurotoxin; toxinology.		
06	03				
19. ABSTRACT (Continue on reverse if necessary and identify by block number) The following report describes the immunization and proprietary plasmapheresis methods used to produce equine hyperimmune botulinal antitoxin plasma. Also included are proprietary methods used for large scale production of an equine botulinal antitoxin with the highest specific neutralizing activity, the lowest potential for sensitization, and consisting of over 95% equine (F(ab') ₂ immunoglobulin. Finally, this report describes the proprietary large scale production and testing of two preparations of equine heptavalent botulinal antitoxin, FPA1-3-45-4x1 and FFA2-3-45-4x1. <i>Reprints</i>					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified		
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia Miller			22b. TELEPHONE (Include Area Code) (301) 663-7325		22c. OFFICE SYMBOL SGRD-RMI-S

Summary

The following report describes the immunization and proprietary plasmapheresis methods used to produce equine hyperimmune botulinal antitoxin plasma. Also included are proprietary methods used for large scale production of an equine botulinal antitoxin with the highest specific neutralizing activity, the lowest potential for sensitization, and consisting of over 95% equine (F(ab')₂ immunoglobulin. Finally, this report describes the proprietary large scale production and testing of two preparations of equine heptavalent botulinal antitoxin, FFA1-3-45-4x1 and FFA2-3-45-4x1.

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DTIC TAB	<input checked="" type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By	
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Availability Code	
Dist	Avail and/or Special
B-3	



FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. [NIH] 78-23, Revised 1978).

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I. Introduction

A. Type of Project

This progress report summarizes the research and production performed under Contract Number DAMD17-82-C-2119, "Research and Preparation of an Equine Heptavalent Botulinal Antitoxin". Hyperimmune plasma was obtained by plasmapheresis of two Army horses: a thoroughbred, First Flight, and a black draft horse, Abe. Experiments and large scale production used only plasma from First Flight. Botulinal toxins A, B, C, D, E, F and G were the antigens used in the hyperimmunization program administered by the Contracting Officer, Major Martin Crumrine, Ph.D. of the United States Army Medical Research Institute of Infectious Diseases (USAMRIID).

B. Technical Objective

The objective of this project was the preparation of a heptavalent equine botulinal F(ab')₂ antitoxin from the hyperimmune plasma obtained by plasmapheresis. The purified antitoxins produced, FFA1-3-45-4x1 and FFA2-3-45-4x1, possessed the following characteristics:

1. Safe for intravenous administration at doses of 30 mg/kg. FFA1-3-45-4X1 has an LD50 of 7.8×10^3 mg/kg. In comparison, the LD50 for a commercial IgG preparation (Cutter Gamimune^R) is 5.00×10^3 mg/kg, and for commercial human albumin (Cutter), 1.13×10^4 mg/kg.
2. Neutralizing activity against botulinal neurotoxins A, B, C, D, E, F, and G.
3. 99% pure equine F(ab')₂ immunoglobulin. Low allergenic activity, having less capacity to sensitize or stimulate antibodies to the antitoxin than current commercial equine botulinal antitoxin.
4. Stable for long-term storage (6 years).
5. Finally, this report constitutes information for an IND and includes details of preparation, specifications of final product, in vitro and animal testing, and establishment of suitable clinical testing.

Plasma from First Flight was pooled and fractionated by proprietary methods developed in this laboratory which can produce 99% pure F(ab')₂ equine antitoxin. The methods involved the following:

1. Treating the hyperimmune plasma with SiO₂ to stabilize the plasma.
2. Pepsin proteolysis at 45°C, pH4.0 for one hour.
3. Removal of aggregates by centrifugation at 32,000 X G.
4. Removal of low molecular weight (MW) peptides resulting from the pepsin hydrolysis.
5. Isolation of the neutralizing F(ab')₂ antibody with ion exchange resin.
6. Sterile "bulk" bottling of the final product in 500 ml and 20 ml bottles. Final sterile bottling in 20 ml bottles, following determination of neutralization activity, and labelled with the appropriate labels indicating expiration date, neutralizing activity for botulinal toxins A, B, C, D, E, F and G, and lot number.

The proprietary methods developed with the support of this grant are the subject matter of a patent application, which will be submitted with all appropriate information when completed.

C. Hypothesis

Life-threatening intoxication by botulinal toxins can result from ingestion of food containing preformed quantities of botulinal neurotoxin and laboratory accidents involving personnel working with botulinal toxins. Intoxication can also result from in vivo toxin production, as is the case for wound botulism (spores colonize a wound) and infant botulism (spores colonize the gastrointestinal tract). Seven specific botulinal toxins have been identified and these include A, B, C, D, E, F and G. While not all have been associated with producing food botulism, those laboratory personnel working with the seven types require a heptavalent antitoxin preparation which currently is not commercially produced or available.

To produce symptoms associated with botulinal intoxication, the toxin must pass from the gut, wound, or accidental injection site into the vascular system, through the capillary endothelial walls, into the tissue spaces and bind with receptors on nerve endings. Following this initial interaction with the nerve endings, the toxin receptor complex is then internalized by adsorptive pinocytosis and when intracellular, a final lytic step completes the sequence resulting in a neuromuscular transmission block (1,2). The strategy in antitoxin therapy is to specifically neutralize all free toxin to prevent toxin receptor interaction since once toxin has combined with the receptor, antitoxin is presumed to be without effect. There is one primary stage in the passage of the toxin from the tissues to the nerve endings that antitoxin, were it present in sufficient quantity, could effectively neutralize the largest quantity of toxin, and that is in the blood. Intravenous administration of antitoxin would offer the best possibility of neutralizing the most toxin in the shortest time period. Therefore, an intravenous antitoxin with the lowest potential for allergic sensitization was the preparation of choice.

Finally and somewhat speculative, is the neutralization of free toxin once it has passed into the tissues. This would require that the antitoxin molecule or a neutralizing fragment pass the vascular barrier. Whether a modified antitoxin molecule might pass remains to be demonstrated. There has been some thinking that toxin neutralizing Fab or $F(ab')_2$ fragments might, because of the lower molecular weight, pass the tissue barrier. Both Fab and $F(ab')_2$ preparations were experimentally produced by this lab. Large scale production produced two $F(ab')_2$ preparations, FFA1-3-45-4x1 and FFA2-3-45-4x1.

D. Background

1. Basis

Classically, botulism constitutes an intoxication rather than an infectious disease and treatment is directed toward removing unbound toxin from the circulation with a specific antitoxin. Pathogenic C. botulinum are normal soil inhabitants, with little or no invasive power. The disease they produce results from the production of a highly toxic protein neurotoxin. Therefore with the possible exception of infectious infant and wound botulism, botulism is an intoxication resulting from the ingestion of foods in which the organism has previously developed and formed a neurotoxic exotoxin, rather than an infectious disease such as diphtheria or tetanus. Outbreaks are rare, but when they occur, require both prompt prophylaxis and therapeutic treatment with antitoxin (3 and 4). Those undoubtedly at highest risk today would be persons involved in research on C. botulinum neurotoxins. It is also conceivable that a hostile element could use these potent neurotoxins to selectively contaminate food supplies.

Unlike the lethal exotoxins in diphtheria and tetanus where there is but a single type specific toxin for each organism, there have been seven types of C. botulinum (A-G) identified, each elaborating an immunologically distinct form of neurotoxin. Each type, therefore, requires its specific antitoxin for neutralization. Not all have been associated with disease in man, however, type G, which was identified in 1969 (5) and had not been associated with mortality in man, was recently implicated in several incidences of sudden death in Switzerland (6). Those working with the various botulinum toxins would be at risk regardless of whether the specific toxin had been previously associated with disease in man.

Since botulism classically is not an infectious disease, but an intoxication, treatment is directed toward neutralizing and removing unbound toxin from the circulation with either specific or polyvalent type A, B and E antitoxins. At the present time a heptavalent antitoxin capable of neutralizing types A, B, C, D, E, F and G toxins is not available. Therefore, for those working with various botulinum toxins including G, or those suspected to have ingested G toxin, there exists no antitoxin for prophylaxis and treatment.

It was the purpose of this project to prepare the most safe and effective equine heptavalent, botulinum antitoxin for clinical use in the treatment and prophylaxis of botulinum intoxication.

2. Previous Work

Biological Immunosuppressant Equine Antilymphoblast Globulin:

This laboratory has been preparing highly purified, native, biologically active, animal IgG for clinical use since 1972. While these heterologous preparations include goat and rabbit IgG, equine antilymphoblast and equine antithymocyte globulin (IgGab) constitute the major biologics prepared. Our equine antilymphoblast globulin has been used clinically in multicenter trials and is currently under consideration for licensing by the Bureau of Biologics. Over one hundred transplant centers use this preparation. This laboratory produces approximately 40,000 gm/yr for clinical use.

This current production of highly purified equine antilymphoblast IgGab required the development of effective immunization, plasmapheresis, and fractionation methods. Most important was the timing of immunizations and plasmapheresis so as to harvest the plasma during the time period when the biologically active (immunosuppressive) IgG was being produced. The schedules and methods evolved have permitted us to maintain approximately 15 horses on a schedule where a total of approximately 84 liters of active plasma can be consistently obtained/horse over a 5 week period. This laboratory has pioneered the development of complete large scale ion exchange chromatographic purification of equine IgGab. The method of plasma fractionation has been demonstrated to consistently isolate the "immunosuppressive" IgGab antilymphoblast antibody. This 99% pure monomeric equine IgG is administered intravenously in doses of 20-30 mg/kg daily for 14 days (7). Allergic manifestations are mild and have been less than 5% (7). In fact, the same preparation can be safely administered for a second course to the same patient to treat rejection episodes (8). However, the transplant patient also receives steroids and Imuran, drugs which are immunosuppressants and reduce the allergic potential of the intact equine IgG.

Equine Heptavalent Botulinal Antitoxin:

Equine antitoxin would be used in individuals not receiving either steroids or Imuran and therefore an antitoxin with decreased allergic potential was needed. Such a preparation would be an F(ab')₂ fragment of the equine immunoglobulin molecule.

The preparation of equine botulinal antitoxin also required some modification in the methods used for preparation of horse antilymphoblast globulin. Whereas the most active (immunosuppressive) horse antilymphoblast plasma is synthesized during a three week period after the second immunization with lymphocytes, high botulinal toxin neutralization activity requires repeated immunizations (~ 30) over a 1-2 year period to achieve high neutralizing activity against the 7 botulinal toxin types (A, B, C, D, E, F and G).

3. Production of Equine Heptavalent Botulinal Antitoxin

The following report describes the immunization and plasmapheresis methods used to produce equine hyperimmune botulinal antitoxin plasma. Also included are the experiments which resulted in the method used for large scale production of an equine botulinal antitoxin with the highest specific neutralizing activity, the lowest potential for sensitization, and consisting of over 95% equine (F(ab')₂ immunoglobulin. Finally, this report describes the large scale production and testing of two preparations of equine heptavalent botulinal antitoxin, FFA1-3-45-4x1 and FFA2-3-45-4x1.

II. Materials and Methods

A. Immunization of Horses

For the purpose of producing equine heptavalent botulinum antitoxin, two horses were repeatedly immunized with botulinal toxoids and toxins. Immunization was begun with toxoids while the horses, First Flight and Abe, were at Fort Detrick, Frederick, Maryland. After antibody neutralizing activity titers were boosted to protective levels, the horses were then immunized with botulinal toxins.

First Flight, a black thoroughbred, was immunized seventeen times with toxoids over a seventeen-month period before immunization with toxins began. Abe, a large draft horse, was immunized with toxoids only ten times before adequate circulating levels of neutralizing antitoxin appeared and then toxin immunizations began at Fort Detrick. First Flight received 33 injections of toxoids and/or toxins before being shipped to the University of Minnesota in November, 1980 (See Table I, Immunization Schedule for "First Flight"). Abe was immunized 13 times with toxoids and/or toxins before being shipped to Minnesota in November, 1982 (See Table II, Immunization Schedule for "Abe"). These horses were shipped to the Minnesota ALG Program for the purpose of large scale plasmapheresis and subsequent fractionation and purification of intact or despeciated equine IgG, heptavalent botulinal antitoxin preparations, in fulfillment of Contract Number DAMD17-82-C-2119.

After arriving at the University of Minnesota each horse was boosted with toxins or toxoids by U.S. Army personnel, either Lt. Col. George E. Lewis or Major Martin Crumrine. To elevate and maintain high neutralizing activity titers the horses were immunized every forty to 100 days. Each horse was plasmapheresed eight times between immunizations.

While at the University of Minnesota these horses were fed specially formulated high protein grain pellets and also a daily ration of hay. Routine health care maintenance, including floating teeth, hoof trimming, grooming, deworming, and tetanus and influenza vaccines were performed as the need arose. The immunization and bleed schedule procedures produced consistently high titer antitoxins without sacri-

ficing the general well being of the animals. The horses maintained high plasma protein and equine IgG levels throughout the rigorous plasmapheresis schedule.

B. Plasmapheresis

Plasmapheresis of First Flight (FF) and Abe followed the current standard procedure, described below:

During plasmapheresis the volume of plasma collected from each horse is a significant percent of the animal's total blood volume, 10-15%. Initially, 20-25% of the whole blood volume is removed, but, by saline fluid replacement and red cell reinfusion, this loss is reduced to manageable levels.

Each horse is plasmapheresed eight times, beginning on the ninth day post immunization. Abe and FF were bled on days 9, 11, 13, 15, 22, 24, 26, 28. The seven day rest period between series (1 series=4 bleeds) allows for stabilization and replacement of body fluids and serum proteins.

The plasma is collected as follows, observing sterile or aseptic technique throughout the process:

The area around the jugular groove is shaved, then triple scrubbed alternately with betadine and 70% alcohol. A tourniquet is tightened to sufficiently "raise" the jugular vein. An upward stick into the jugular vein is made with a 3", 10 gauge needle (#11040 Popper & Sons, New Hyde Park, NY). The needle is attached, with silastic tubing (#601-405, Dow Corning, Midland, MI), to a custom-designed polypropol bleed bottle (Nalgene Labware Division, Rochester, NY) containing the citrate. The bleed bottle, with citrate and attachments, is steam autoclave sterilized prior to use.

As the blood flows into the jug it is mixed with the citrate by repeated agitation during the bleeding. Bleeding continues until a total volume of 12 liters is collected (citrate volume + blood volume). At that time, the tubing is clamped and disconnected from the needle at a plastic tubing connector attached just below the needle, and the bleed bottle is placed in a settling rack to allow settling of the red blood cells; the needle is not removed from the vein. A polypropylene jug (#2220-0020, Nalgene) containing 7 liters of sterile normal saline is hung above the horse and attached, by tubing, to the needle. The tubing is unclamped and 4 liters of saline is infused into the horse. This immediate fluid replacement reduces the incidence of shock. The needle is then removed, the jugular groove area rinsed with water, and the horse is returned to its tie stall while the red blood cells (RBCs) settle. Due to rouleaux formation, the RBCs settle out within 4-6 hours. After settling at ambient temperature, the RBCs are carefully drained, through tubing at the bottom of the bleed bottle, into another bottle containing approximately 3 liters of saline for reinfusion. The saline and RBC's are mixed well and hung above the horse. The horse is again triple scrubbed with betadine and 70% alcohol and a tourniquet is applied. A downward stick

is then made into the jugular vein with a sterile 3", 10 gauge needle (#11040, Popper & Sons). The saline bottle tubing is then attached to the needle, unclamped, and the horse reinfused with the entire saline/red cell mixture. When reinfusion is complete, the needle is removed, the jugular groove area rinsed with water, and the horse is returned to its stall.

The plasma is refrigerated overnight until the residual red blood cells are removed by centrifugation (see Plasma Processing and Storage). The formulations for the sodium citrate anticoagulant and reinfusion saline are as follows:

Sodium Citrate (per liter)

3.18 grams citric acid
38.00 grams sodium citrate
4.70 grams sodium phosphate (dibasic)
1 liter non-pyrogenic water
pH 6.18 - pH 6.22

Reinfusion Saline (per liter)

8.76 grams sodium chloride
1 liter non-pyrogenic water
pH 5.6 - pH 6.0

C. Plasma Processing and Storage

Plasma collected from First Flight and Abe was processed as follows. To prepare it for long-term storage, plasma is processed within 36 hours of plasmapheresis. The plasma is poured from the bleed collection container into 1 liter sterile, polypropylene centrifuge bottles. The plasma is spun in refrigerated swinging bucket centrifuges to remove any remaining cells or tissue. A list of the centrifuges used, and the operating parameters follows.

<u>Centrifuge</u>	<u>Rotor</u>	<u>Speed</u>	<u>RCF</u>	<u>Temp. Setting</u>	<u>Time</u>
Dupont-Sorvall RC3	HG-4	5000RPM	6975 X G	4°C	20 min.
Dupont-Sorvall RC3B	H6000A	5000RPM		4°C	20 min.
Beckman J6	JS 4.2	4200RPM	5010 X G	0°C	30 min.

E.I. Dupont de Nemours and Co., Newton, CT
Beckman Instruments, Palo Alto, CA

The plasma is then measured out in a 4 liter graduated cylinder and poured into sterile 4 liter polypropylene bottles for storage.

One or more 5 ml samples of Abe and FF plasma were taken for determination of protein concentration and neutralizing activity. Sample volumes of 250 ml were set aside from some bleeds to prepare experimental plasma pools. Each container of plasma was labelled with a red

bordered label for FF, or a blue bordered label for Abe. The label contained the name of the horse, bleed number, date of plasma processing, volume of plasma in the containers, container designation (A, B, C. . .), and the initials of the processor. The plasma was frozen at -20° C and then transferred to a -20°C storage facility for long-term storage.

D. Experimental Fractionation

1. Source of Plasma

Experiments in this report were performed with plasma from the antitoxin horse, First Flight.

The plasma source for early experiments was First Flight bleeds 1-33. The only exception was final product 7 when SP1 bottled material (Bleeds 10, 14, 18, 22, 26 and 30) was used. All other experiments began with Pool A First Flight Bleeds 1-73, excluding 46-49, as the starting material.

2. Preparation of Buffers

Various buffers were used during fractionation of equine protein primarily to adjust the pH, conductivity, and solvent composition of the protein solution. All buffers were prepared in autoclaved polypropylene containers. Water used for buffer preparation was deionized, distilled, sterile and pyrogen-free. Each buffer was tested for pH and conductivity, and found to be within specified limits before used. The acceptable range for pH was ± 0.05 units, and for conductivity, ± 0.1 mS. Buffers used in equilibration and running of anion exchange columns, or for protein reduction, were deaerated by vacuum aspiration prior to use. Below is a list of buffers used in the experimental procedures.

<u>Buffer Name</u>	<u>pH</u>	<u>Conductivity at 21°C (mS)</u>	<u>Composition</u>
imidazole acetate	6.60	5.85	19mM imidazole, 89mM acetate
sterilizing buffer	7.00	2.05	13mM imidazole, 60mM acetate, 30% ethanol
glycine saline	6.80	12-15	*300mM glycine, 150mM sodium chloride
normal saline	-	-	**0.9% sodium chloride (150mM)
pepsin activating buffer	3.00	-	3 parts .01M HCl plus 2 parts .01M NaOH
phosphate buffered saline	8.00	-	100mM phosphate, 100mM NaCl
sodium acetate	4.00	6.70	540mM acetate
sodium acetate	5.00	6.40	17mM imidazole, 140mM acetate
Trisma base	-	-	saturated solution at room temp.

*Adjusted to pH 6.8 with 5N NaOH.

**Adjusted to pH 7.2 with 0.1N NaOH.

3. Stabilization of Equine Plasma by SiO₂ Treatment

Treatment of plasma with SiO₂ stabilizes the plasma by eliminating fibrinogen, plasminogen and a number of easily denaturable proteins. When silica powder (Aerosil 380, Degussa, Inc., Tetaboro, NJ) is added to plasma, certain plasma components are preferentially adsorbed onto the surface of the silica particles. The particle and adsorbed protein complex are then removed by sedimentation. Some proteins, including fibrinogen, are so efficiently bound as to be quantitatively removed by this procedure. Removal of these proteins results in a highly stable, clarified protein solution. Some of these proteins, if not removed by SiO₂ treatment, would hinder subsequent plasma fractionation by anion exchange chromatography since they would elute with the equine antibody fraction to be isolated.

Silicon dioxide is a sterile, non-crystalline, pyrogen-free, dry powder which is prepared by the reaction of silicon tetrachloride in a oxy-hydrogen flame. Dry silicon dioxide was slowly added to equine plasma, with mixing, to a final concentration of 15 gms SiO₂ per liter of plasma. The plasma and SiO₂ were mixed with the aid of motor driven rotors for one hour at 4°C. The solution was then centrifuged at 27,000 X G, 4°C for 45 minutes (RC2-B, Sorvall-Dupont Instr), and the clarified supernatant filtered through sterile gauze. The precipitate was discarded.

4. Clotting of Equine Plasma

Clotting, by addition of calcium, was done to remove fibrinogen from plasma; this procedure stabilizes the plasma. The exact amount of calcium (3.5 M CaCl_2 aq.) added to citrated horse plasma is critical for the formation of a rapid clot. This amount was determined experimentally on a small sample of the horse plasma to be clotted. In general, a rapid clot was formed when 0.5 to 1.0 ml of 3.5 M CaCl_2 (aq.) per 100 ml of horse plasma was added. After adding calcium to the plasma, the solution was mixed thoroughly while raising its temperature rapidly to 37°C in a 56°C water bath. The solution was then transferred to a 37°C incubator for 2-6 hours. The clot was rotated away from the sides of the bottle occasionally. Following incubation, the clot was removed by centrifugation (45 min./4°C/5,000 X G).

5. Preparation of Pepsin

Pepsin Activation: Porcine pepsin (2 X crystallized, lot #516360, 2460 units/mg, Calbiochem Behring, La Jolla, CA) was dissolved in 0.01N HCl/NaOH, pH 3.0, to a final concentration of 10 mg/ml. The solution was then mixed at room temperature for 10 to 30 minutes. Activation of pepsin is done immediately prior to use.

6. Ammonium Sulfate Precipitation

The Japanese method for pepsin despeciation of protein requires precipitation of the plasma with ammonium sulfate; this protects the immunoglobulins from denaturation due to the high temperatures used during this procedure.

Ammonium sulfate crystals (Grade I, #A-5132, Sigma, St. Louis, MO) were added to the plasma at a ratio of 150 grams $(\text{NH}_4)_2\text{SO}_4$ per liter of volume, making a 26% saturated solution. The solution was mixed and then centrifuged at 20°C, to separate the precipitated protein and the supernatant. To recover any unprecipitated protein, the supernatant was then brought to a final ammonium sulfate concentration of 50% by the addition of 158 gm $(\text{NH}_4)_2\text{SO}_4$ per liter of volume, and this solution was mixed and centrifuged at 20°C. Again, the supernatant from this centrifugation was brought to a final ammonium sulfate concentration of 50% by the addition of 158 gm $(\text{NH}_4)_2\text{SO}_4$ per liter, mixed for two hours at room temperature, and centrifuged at 20°C. The precipitates (protein) were resuspended in sterile, distilled water.

7. Heat Exchange

Pepsin despeciation of protein requires a warm protein solution. Heating of protein solutions was accomplished by heat exchange through a stainless steel coil. A description of the heat exchange system follows; see Figure 1 for a diagram of the system. The coil used in experimental procedures was made of stainless steel tubing (0.25" O.D., 0.19" I.D.) shaped into twelve loops. The overall dimension of the coil was 11 cm in diameter by 18 cm

in height. Prior to use, the coil was cleaned with soap and water, rinsed with alcohol and then rinsed with non-pyrogenic water. The heat exchange coil was then placed in the protein solution (1 to 2 liters final volume) contained in a 4000 ml Pyrex beaker (No. 1003). Mixing was provided by a teflon, magnetic stir bar. Six gallons of water were heated to the highest temperature needed during the experiment (37-58 degrees C) by an immersion circulator (Polystat 22, Cat. #1252-00, Cole Parmer, Chicago, IL) which maintains a set temperature ± 0.1 degrees C. The heated water was recirculated through the heat exchange coil via a pump (Gear pump, Cat. #12A-41-316, Micro Pump, Concord, CA) at a rate of 2 liters per minute. Within 8-10 minutes, the protein solution was the same temperature as the six gallons of warm water. To cool the protein solution, ice water was pumped through the coils.

8. Centrifugation

Centrifugations during experimental fractionation procedures were performed in a Sorvall Superspeed RC2-B automatic centrifuge (RC2-B, Sorvall-Dupont Instruments, Newton, CT) equipped with a type GSA fixed rotor. The rotor capacity is 2 liters, in six containers, and the maximum radius is 5.75 inches. Centrifugations were performed at 13,000 RPM for 45 minutes. At the maximum radius, the effective force is 27,000 times the force of gravity. Centrifugations were carried out at 4°C, with the exception of centrifugations just prior to anion exchange chromatography which are performed at 20°C.

9. Concentration and Diafiltration

The protein and buffer composition of the equine plasma was adjusted prior to anion exchange and bottling by concentration and diafiltration, using an ultrafiltration system. The system consists of a low hold volume molecular filtration cell (Pellicon Ultrafiltration System, Millipore Corp., Bedford, MA) equipped with 5 square feet of type PTGC (Millipore) 10,000 MW cut off membrane. The system is fed by a peristaltic pump with back pressure less than or equal to 20 psi.

Concentration - The total protein concentration was increased by recirculating the protein solution through the ultrafiltration system under pressure, and discarding the filtrate, until the desired protein concentration (20-60 mg/ml) was reached. Small molecular weight molecules crossed the membranes and were eliminated as filtrate, resulting in the concentration of proteins larger than 10,000 daltons.

Diafiltration - After the desired protein concentration was reached, buffer was pumped into the retentate at a rate identical to the rate of filtrate removal. This resulted in a constant retentate volume and a constant protein concentration. This process of simultaneous concentration and dilution continued until enough buffer was added to equal 7-10 times the retentate volume, ensuring the replacement of 99% of the original protein solvent with buffer. Diafiltration was used in place of dialysis because

it is an extremely rapid method and it allows for the manipulation of protein concentration.

Ultrafiltration was also used, in the case of pepsin digested equine protein, as a purification technique to remove low molecular weight proteins and protein fragments.

10. Anion Exchange Chromatography

Anion exchange chromatography of equine plasma, or pepsin treated plasma, was used to isolate the active immunoglobulin fraction. Two methods of anion exchange were performed, batch and column. Under the proper conditions of pH and conductivity (pH=6.6, conductivity at 21°C=5.85 mS), non-immunoglobulin protein molecules are negatively charged and bind to the anion exchange resin; the immunoglobulin fraction, which is neutral or positively charged, does not bind and, in the case of column chromatography, is eluted in the first peak. Chromatography was performed at room temperature.

Batch Anion Exchange:

A batch procedure was used, after pepsin despeciation, to bind and remove pepsin from the plasma (pepsin pI=1.0). Pepsin would slow column flow rates, by binding to QAE, if not removed by this batch procedure. Dry QAE A-50 (Lot #8578, Pharmacia Fine Chemicals, Uppsala, Sweden) was added to a final concentration of 1.25 gm QAE for each 1.00 gm pepsin used in the experiment. The QAE and plasma solution was mixed for 45 minutes at room temperature. The QAE was then removed by filtration through gauze.

Column Chromatography:

Column Preparation - The anion exchange resin, QAE-A50, (Lot #15446, Pharmacia), was initially allowed to swell in sterilizing buffer. The pH and conductivity of this alcohol buffer was chosen so that the resin would swell to the same extent in this buffer as in the non-alcohol buffers used subsequently. Small fines, impurities in the resin, were removed by allowing the resin to settle and then decanting and discarding the top layer of alcohol buffer which contained the fines. This process of defining was performed two times and effectively removed the lightweight fines from the resin. The swollen resin was then loaded into a column. The amount of resin used was determined by the total amount of protein to be applied; resin was used for each 0.4 to 0.8 grams of protein. The amount of protein applied per gram of resin is called the "load". The resin is used once and then discarded.

Column Equilibration - Five column volumes of pH 6.6 buffer were pumped through the loaded column at a rate of 0.2 to

0.5 column volumes per hour. This was done to remove the alcohol and to equilibrate the column to the proper conditions of pH and conductivity. A column with a volume of 500 ml requires 2.5 liters (5 X 500 ml) of pH 6.6 buffer to be applied before the proper conditions are met.

Protein Application - Diafiltered protein solution was applied to the column at a rate of 0.2 to 0.5 column volumes per hour with a peristaltic pump (Micropex, LKB). The column eluant was collected in fractions of 10-12 ml (Multirac, LKB, Model 2238) and the absorbance at 280 nm was measured and recorded, (U.V. monitor, 0.5 mm flow cell, 2.0 AUFS, LKB; Model 2210 recorder, LKB). For most experiments, one peak at pH 6.6 was collected. For some experiments, a second and third peak were eluted with pH 5.0 and pH 4.0 buffer respectively. Peaks were collected until the protein concentration dropped below 0.8 mg/ml.

11. Reduction and Alkylation

Reduction and alkylation were used to produce Fab fragments from equine F(ab')₂.

Reduction:

Cysteine-HCl (C-9768, Sigma, St. Louis, MO) was dissolved in pH 8.0 PBS buffer (100mM phosphate, adjusted to pH 8.0 with 1 N NaOH). PBS was used to dilute the cysteine to a concentration of 1.0 M.

Equine F(ab')₂ was diafiltered against pH 8.0 PBS and concentrated to 30-35 mg/ml. The concentration of dissolved oxygen in the F(ab')₂ and cysteine solutions was reduced by vacuum aspiration. The protein, cysteine and pH 8.0 buffer solutions, all deaerated, were added together to a final concentration of 20 mg/ml F(ab')₂ with 0.2 M cysteine. The solution was heated to 37°C, with gentle mixing, for one hour.

Alkylation:

Alkylation was accomplished by the addition of iodoacetamide (I-6125, Sigma) immediately following reduction. Iodoacetamide, 0.2 M in pH 8 PBS, was added to the reduced protein to a final concentration of 0.02 M; this was roughly a 50 fold excess of iodoacetamide since the molar concentration of Fab (45,000 to 49,000 daltons) at 20 mg/ml is around 0.0004 M. Diafiltration against 10 volumes of pH 8 PBS containing 0.01 M iodoacetamide removed the cysteine added during reduction. Iodoacetamide was then removed by diafiltration against 10 volumes of pH 6.8 glycine-saline buffer. The Fab final product was concentrated, (ultrafiltration system) centrifuged (27,000 X G, 4°C, 45

minutes), and sterile filtered in preparation for sterile bottling in 10cc vials.

12. Sterile Filtration

Prior to bottling, all experimental final products are sterilized by vacuum filtration through a 0.22 micron, 150 ml filter unit (#7103, Falcon, Oxnard, CA).

E. Experimental Testing

The following assays were used to analyze the experimental plasma and final products.

1. Biuret

Purpose:

Total protein, in experimental plasma and product samples, were determined by the Biuret method.

Procedure:

The sample or standard, 50 μ l, is added to 5.0 ml of normal saline. Biuret reagent (13.5 mM CuSO_4 , 7.84 N NaOH), 2.5 ml, is added to the diluted protein, mixed, and incubated at room temperature, for 20-60 minutes. Following incubation, the absorbance at 310 nm (Model 2600 spectrophotometer, Gilford, Oberlin, OH) is determined. A four point standard curve is constructed using bovine serum albumin standards (Cat. #81-016, Miles Labs., Inc., Elkhart, IN) at 20, 40, and 60 mg protein/ml, and a blank consisting of 5.0 ml saline and 2.5 ml of biuret solution. All samples and standards are tested in triplicate and the average absorbance is used. The protein concentration of each sample is determined by using a linear regression formula based on the absorbance of the four standards.

2. Radial Immunodiffusion (RID)

Purpose:

Individual plasma proteins, in plasma and product samples, were quantitated by the Mancini method (9).

Procedure:

Samples are placed in wells cut in agar which contains specific antibody. When the specific antibody in the agar reaches equilibrium with its antigen, a ring of precipitate is established. At end point, the diameter of the ring is directly proportional to the protein concentration of the unknown, and the protein concentration is then determined by comparison with known

standards. Ring diameters are measured at endpoint, to the nearest 0.1 mm, using a calibrating RID viewer (Cat. #928, Kallestad Labs).

Equine alpha 2-macroglobulin, IgG, transferrin, and albumin have been purified at this laboratory for use as standards and as antigens for the production of antisera. Antisera to equine IgT, IgA and IgG were purchased from Pel Freez Biologicals, Rogers, Arkansas. Horse IgA and IgM standards were purchased from Miles Laboratories, Inc., Elkhart, Indiana. We based our mg/ml IgG(T) on the published reports that normal citrated equine plasma has an IgG(T) concentration of 1-4 mg/ml (10). Custom-made Mancini plates were supplied by M.D. Labs, Richfield, MN.

3. Immunoelectrophoresis (IEP)

Purpose:

The purity and electrophoretic mobility of equine protein samples were analyzed by immunoelectrophoresis.

Procedure:

In immunoelectrophoresis, two methods are combined--electrophoresis of the samples in an agarose matrix, followed by immunodiffusion and reaction with antiserum specific for equine proteins.

Agarose gel (1%) IEP plates (IEP Kit #912, Kallestad Labs., Austin, TX) are used for all sample analyses. These are pre-cut plates, each containing 7 sample wells divided by 6 antiserum holding troughs. Sodium barbitol IEP buffer is also supplied in the kit. This is dissolved in distilled water, pH 8.6.

The IEP plates containing experimental samples are placed on the cooling stage of a multiphor electrophoresis unit (Model #2117, LKB, Gaithersburg, MD). One liter of barbitol buffer is placed into the tanks on either side of the cooling stage. A recirculating cold bath (Model #2209, LKB) is used to keep the cooling stage at 20°C. Samples are placed in the wells, 2 µl per well. Filter paper wicks are used to provide electrical contact between the buffers and the plates. Following electrophoresis at 120 volts (Model #2103 power supply, LKB) for two hours, the gel is removed from the electric field and antiserum, specific for individual equine proteins or for equine serum (Pel Freez Biologicals), is added to the troughs, 100 µl per trough.

The antiserum is allowed to diffuse into the agar and react with the separated specific proteins for 18 hours at room temperature. The plates are then blotted and pressed for two hours, followed by washing in two changes of 0.9% NaCl to remove unprecipitated proteins. They are then soaked for 18 hours in distilled water, to remove the salts; stained with 0.5% Coomassie Blue R-250 stain; destained; and dried.

4. Isoelectric Focusing (IEF)

Purpose:

The isoelectric points or range of proteins in plasma and produced samples were determined by isoelectric focusing.

Procedure:

Samples are focused in a linear pH gradient, from pH 3.5 to pH 9.5, on pre-cast gels (Cat. #1804-101, LKB). The gels contain 2.4% (w/v) ampholyte, 5% (w/v) polyacrylamide, are 3% cross-linked, and 235 X 90 X 1 mm in size. Equipment used includes a multiphor electrophoresis unit (Model #2117, LKB), power supply, (Model #2103, LKB) and recirculating cold bath (Model #2209, LKB).

Twenty samples, 20 µl each containing 0.2 to 2.0 mg protein, are applied onto filter paper tabs (5 X 10 mm, Whatman, Paratex). These tabs are then placed on the gel, approximately 2 cm from the cathode, and the gel is placed on the electrophoresis unit. Filter paper wicks, soaked in the cathode and anode electrode solutions, 1.0 M NaOH and 1.0 M H₃PO₄, respectively, are then placed on the gel, providing the electrical contact between gel and electrode. The samples are focused for 90 minutes, 4°C, at 30 watts constant power; the application tabs are removed after 45 minutes.

At the end of the run, the pH gradient of the gel at 4°C is determined. A Beckman 3500 pH meter (Beckman Instruments, Irvine, CA) with a combination surface electrode (Cat. #2117-111, LKB) is used to determine the pH of the gel in 1 cm increments. The gel is focused for 10 additional minutes to increase the sharpness of the bands. On some gels, pH standards (pH Kit 3-10, Pharmacia, Piscataway, NJ) are included in the gel, and the gel is allowed to reach 20°C during the last 20 minutes of the focusing run. The focused gel is fixed for 45 minutes in a solution of 1% methanol (v/v) and 0.5% sulphosalicylic acid. Prior to staining, the gel is soaked for 5 minutes in destaining solution which contains 25% ethanol (v/v) and 8% acetic acid (v/v). This allows the gel to equilibrate to the staining conditions and also helps to remove ampholytes. The gel is stained for 10 minutes at 60°C in a filtered stain consisting of 0.1% Coomassie Blue R-250 (w/v) in destaining solution. The gel is then destained for at least 48 hours with several changes of destain. To preserve the stained gels, they are soaked in destaining solution containing 10% glycerol (v/v) for 45 minutes, air dried overnight, and covered with a thin plastic film.

5. Preparative Focusing in a Granulated Gel (PEGG)

Purpose:

Preparative focusing was used to separate proteins in plasma samples by isoelectric point, the proteins from discrete isoelectric regions could then be eluted and analyzed.

Procedure:

PEGG is performed in a glass tray (244 X 103 X 5 mm) containing a 100 ml slurry of protein (60-150 mg), highly washed G-75 Sephadex (4.0 grams), ampholytes (5% w/v), and distilled water. The protein is generally dialyzed against 1% glycine, to reduce the salt content, prior to being included in the slurry. The ampholyte mixture is chosen to give a linear pH gradient throughout the pH range of interest.

The slurry is poured into the glass tray and dried, with a 4" muffin fan, to an optimal water content. The cathode and anode wicks are soaked in 1.0 M NaOH and 1.0 M H_3PO_4 , respectively, and are placed along the short sides of the tray.

Samples are focused for 16-18 hours, 10°C, at a constant power of 8 watts. Voltage is set not to exceed 1,200 volts. Equipment used includes a multiphor focusing unit (Model #2117, LKB), power supply (Model #2103, LKB), and a recirculating cold bath (Model #2209, LKB). After the run is complete, a print is taken, as described below, to record the pattern of the completed separation. A sheet of filter paper (Whatman #1, 224 X 103 mm) is applied directly to the gel surface for 30 seconds, removed and quickly dried with a hot air blower. The dry print is washed three times in 10% trichloroacetic acid, 15 minutes each wash. The print is then stained for 10 minutes, at room temperature, in a stain consisting of 0.2% Coomassie Blue R-250 dye in a destaining solution (1:5:1-methanol:water:acetic acid), and destained, in the same solution without the dye, until the background color disappears. The print is then air dried.

The separated protein zones are collected by sectioning the gel bed into 30 equal parts using a fractionating grid (Cat. #90-00-0161, LKB). The pH gradient at 10°C is determined by placing a combination surface electrode (Cat. #2117-111, LKB) directly into each section. Thirty small columns are placed over thirty polypropylene test tubes (Cat. #2006, 17 X 100 mm, Falcon, Oxnard, CA). Each gel section is transferred, using a spatula, to a small column and mixed with 1.5 ml 1% glycine. After the gel bed has settled, an additional 1.5 ml 1% glycine is added to the top of each column. The columns are centrifuged for 60 minutes, 280 X G, 4°C, and the elutions collected in the test tubes. Total protein of each elution is determined by measuring the absorbance at 280 nm (Model 2600 spectrophotometer, Gilford, Oberlin, OH). Each elution may be tested further using radial immunodiffusion,

enzyme assays, immunoelectrophoresis or analytical isoelectric focusing. Typically, 3 ml of eluant is recovered from each column, and the total recovery of protein is 70-80%.

6. High Performance Liquid Chromatography (HPLC)

Purpose:

HPLC was used to determine protein purity of a sample and to estimate the molecular weight(s) of a particular protein or mixture of proteins.

Procedure:

HPLC is performed on a rigid, spherical, porous gel matrix consisting of silica particles, 10 ± 2 micron in size, with a hydrophilic surface.

Ten microliters of sample (7-20 mg/ml) are supplied to two 300 X 7.5 mm TSK-3000-SW columns (Beckman Instruments) connected in series. The columns are equilibrated in PBS buffer, pH 6.3 ± 0.1 , 100 mM phosphate, 100 mM NaCl, at a flow rate of 0.5 ml/minute (Model 112 pump, Beckman Instr.). The column effluent is monitored for one hour at 280 nm (Model #2238 UV monitor, 2.5 mm flow cell, 0.05 AUFS, LKB).

The molecular weight of each peak is estimated using a linear regression formula based on the retention times of globular proteins of known molecular weight ranging from 10,000 to 300,000 daltons. Proteins elute in order of decreasing size. The molecular weight of a sample is reproducible within $\pm 5\%$ over a six month period.

7. SDS Polyacrylamide Electrophoresis

Purpose:

Sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis was used to separate protein subunits and to estimate their molecular weights. Electrophoresis was carried out in a discontinuous buffer system, as described by Laemmli (Laemmli, 1970).

Procedure:

Gels are poured into a 14 X 16 X 1.5 cm glass mold. Each gel consists of 28 ml of a 7.5% acrylamide (2.6% C) resolving gel (pH 8.8, 375 mM Tris, 0.1% SDS) and a 5 ml 3% acrylamide stacking gel; ten application wells, 150 μ l each, are molded into each gel. Equipment used includes a vertical electrophoresis unit (Model #201, LKB), recirculating cold bath (Model #2209, LKB) and a power supply (Model #2103, LKB).

Samples are dissolved to a final concentration of 1 to 2 mg/ml in sample buffer (pH 6.8, 62.5 mM Tris, 2.0% SDS, 10% glycerol, 2 mM

dithiothreitol, 0.1% bromophenol blue), boiled for five minutes and then cooled to room temperature. When an estimate of molecular weight is desired without disulfide bond breakage, the dithiothreitol is omitted.

The gel is immersed in electrode solution (pH 8.3, 25 mM Tris, 192 mM glycine, 0.1% SDS), and electrophoresed at 15°C for five hours at 40 mA constant current. The gel is removed from the mold and simultaneously fixed and stained overnight (0.1% Coomassie Brilliant Blue R-250 w/v, 50% ethanol v/v and 5% acetic acid v/v). The gel is then destained (40% ethanol v/v, 3% acetic acid v/v) until the background is clear of stain. To preserve the gel, it is then soaked for one hour in destain containing 10% glycerol (v/v). The gel is air dried overnight, or dried in one hour using a slab gel drier (Model #SE-540, Hoefer Scientific Instr., San Francisco, CA). Molecular weights are estimated by measuring the distance of protein migration relative to the dye front and then comparing these mobilities with those of known marker proteins; a linear relationship exists between the logarithm of a subunit's molecular weight and its mobility (Weber, 1969 and Shapiro, 1967).

8. Ouchterlony

Purpose:

Ouchterlony analysis was used to test PEGG samples for the presence or absence of certain proteins. Specific proteins found were then quantitated by radial immunodiffusion.

Procedure:

A buffered, agarose gel is used as the support medium for this double diffusion technique. Using a three-well system, the anti-serum (against horse serum, IgG, IgA, IgM, IgG(T), transferrin, alpha₂ macroglobulin, or albumin) is placed in the bottom well and the upper two wells contain the samples for qualitative evaluation. Following a 48 hour incubation period, at room temperature, the gels are rinsed with two changes of 0.9% NaCl (2 hours each) to remove unprecipitated proteins. They are then rinsed overnight in distilled water to remove the salts; dried; stained with 0.5% Coomassie Blue R-250 stain; destained; and dried.

9. Conductivity and Measurements

Purpose:

Samples of buffer and protein solutions are tested to ensure conductivity falls within an established acceptable range.

Procedure:

Conductivity, of buffer and protein solutions, is measured on a standardized meter (Model CDM3, Radiometer, Copenhagen, Denmark). Standard conductivity solutions are used to calibrate the meter. Conductivity measurements always include the temperature of the sample during the measurement. Results are expressed in milli siemens (mS).

10. pH Measurements

Purpose:

Buffers and protein solutions are sampled and tested to ensure the measured pH falls within an established, acceptable range. PEGGs are also tested for pH to assist in protein identification.

Procedure:

Measurements of pH are performed using a Beckman pH meter (Model 3500, Beckman Instr.) with automatic temperature compensation. The accuracy of these measurements is optimized in two ways. First, the meter is always calibrated with standard solutions of the same temperatures the sample to be measured. Second, different types of electrodes are chosen to optimize different applications. For pH determinations of PEGG samples at 10 degrees C, a special surface electrode (Cat. #39505, Beckman Instr.) previously standardized at room temperature is used. A combination electrode with a fast junction flow rate (E-4503, Sigma, St. Louis, MO) is used for all despeciation experiments; the fast junction flow rate is needed to prevent clogging of the liquid junction. In addition, the electrode is standardized at the highest temperature (37°C to 58°C) which the plasma will reach during each experiment.

11. Neutralizing Activity

The antitoxin neutralizing activity of samples was determined at USAMRIID by Lt. Colonel George E. Lewis and Major Martin Crumrine. Samples were shipped to Fort Detrick shortly after bottling to be tested for in vivo neutralizing activity against toxin types A, B, C, D, E, F and G. A description of the procedures used for testing can be obtained from Major Martin Crumrine of USAMRIID.

F. Fractionation - Production

The methods used in the large scale production of equine antitoxin F(ab')₂ were based on procedures developed in this lab through small scale fractionation experiments. These experiments are described and discussed elsewhere in this report. This fractionation-production section details the materials and methods used in large scale production in a sequential fashion. Flow Sheet 1 shows this sequence.

To prevent and control bacterial contamination of the plasma during fractionation, certain general procedures were observed. All containers, utensils, instruments, and equipment in contact with the product or buffers were sterilized, by steam autoclaving or with 70% ETOH, or sanitized with 1% bleach followed by repeated rinses with 80°C pyrogen-free water. The product was maintained at 4°C except during anion exchange, despeciation, and bottling procedures which require brief warming. All personnel wore clean scrub suits, gloves and bonnets, and exercised clean or aseptic techniques.

1. Plasma Source - Preparation of Bleed Pool A

To provide a homogenous source of starting material for several production runs, plasma from a large number of First Flight bleeds was combined and designated "Bleed Pool A". Bleed Pool A was prepared by pooling the available plasma from bleeds 1-73. Some of the plasma from these bleeds had been removed from inventory and used for experimental or special lot production. For example, Bleeds 46-49 were used to produce lot FFA30. The volume of plasma from each bleed used in Bleed Pool A is shown in Appendix 1.

The individual bleeds were thawed to 4°C and combined in a 150 gallon polyethylene tank that had been sanitized with 1% bleach followed by repeated rinses with 80 degree C pyrogen-free water. The plasma was mixed by recirculation with a 10 gallon per minute sanitary pump (Model LP30 Amicon Corp., Danvers, MA). The plasma was then dispensed into 4 liter sterile polypropylene containers, labelled, and returned to the cold storage facility for freezing and storage at -20 degrees C. Each red bordered label contained the title, "FF Bleed Pool A", container number, volume, and date.

2. Preparation of Lot Plasma Pools

At the start of a lot production run, selected frozen containers of pooled plasma were thawed to 4°C. The thawed plasma was combined in a 30 or 50 gallon tank. All containers, equipment and instruments in contact with the plasma were previously sterilized by steam autoclaving or EtOH, or were sanitized with 1% bleach followed by repeated rinses with 80°C pyrogen-free water. The plasma was mixed and sampled, and the volume recorded.

3. Stabilization of Plasma

Addition of Silicon Dioxide (SiO₂) - Sterile, pyrogen-free, synthetic SiO₂ (Aerosil 380, Degussa, Teterboro, NJ) was slowly added in a dry form to the plasma pool, while mixing the plasma rapidly. Care was taken to avoid foaming. After the desired amount of SiO₂ had been added, 10-30 grams of SiO₂ per liter of plasma, the mixture was stirred for one hour and then allowed to settle overnight at 4°C.

Removal of SiO₂ - SiO₂ was removed from the plasma by centrifugation. After settling in a tank overnight, the top layer of the plasma-SiO₂ mixture (the supernate) contained very little SiO₂.

The supernate was pumped into a second tank for a later high speed centrifugation step. The bottom layer in the settling tank (the sediment) contained most of the SiO_2 and some plasma. This sediment was dispensed into 1 liter, sterile, polypropylene bottles for low speed centrifugation. This low-speed spin was done either in Dupont-Sorvall RC3 refrigerated centrifuges with HG-4 swinging bucket rotors (E.I. Dupont de Nemours and Co., Newton, CT) at 4°C , 5000 RPM (6975 X G) for 45 minutes, or in a Beckman J-6 refrigerated centrifuge with a JS 4.2 swinging bucket rotor (Beckman Instr., Palo Alto, CA) at 4°C , 4200 RPM (5010 X G) for 60 minutes. The supernate from this spin was added to the supernate drawn off earlier from the settling tank. The pellet from the low-speed spin, packed SiO_2 , was discarded.

A final high speed centrifugation step was done to remove residual SiO_2 particles. The combined supernates were pumped into a Beckman J2-21C, J2-21 or a J2-21M refrigerated high speed centrifuge, equipped with a JCF-continuous-flow rotor, spinning at 18,000 rpm (32,260 X G), 4°C , at a feed rate of 250 ml/min/rotor.

4. Buffer Preparation

Buffers and salt solutions were used to adjust or control pH, conductivity, and ionic composition of the protein solutions at various stages of production. Buffers were also used to equilibrate the ion exchange resin. All buffers and salt solutions were prepared in autoclaved polypropylene tanks, or in polyethylene tanks sanitized with 1% bleach and rinsed six times with 80°C pyrogen-free water.

Water for preparation of buffer was purified by deionization, followed by distillation in a Barnstead 30 gallon per hour Century Cyclone still (Barnstead Co., Boston, MA) with storage at 80°C , making it sterile and pyrogen-free.

5. Sterile Filtration - Buffers and Plasma

Buffers were sterile filtered prior to use by pumping through an autoclaved 0.2 micron absolute membrane filter (Pall DFA 3001ARP or Pall FLF 6001 NR², Trinity Micro Corp., Cortland, NY).

The plasma was filtered twice during fractionation, once after stabilization and once after despeciation, to remove any bacteria introduced during plasma pool preparation or during heat exchange procedures. Plasma filtration was accomplished by placing the plasma in stainless steel pressure vessels, pressurizing to 10-20 psig with medical grade nitrogen (nitrogen N.F., Ohio Medical Products, Madison, WI), and feeding the pressurized plasma to the filtration system.

The filtration system consists of a 20 inch prefilter (Pall AB2NA7P, or Gelman Preflow NFR, Gelman Sciences, Inc., Ann Arbor, MI) in a stainless steel housing of sanitary design (Pall

SANN2G723) followed by a 10 inch 0.2 micron absolute membrane filter (Pall AB1NR7P) in a stainless steel sanitary housing (PALL SANN1G723). The first filter was used to remove most of the bacteria and prevent the second filter from plugging. The second filter, which was steam sterilized before use, removed all remaining bacteria. If the plasma was to be further processed immediately after filtration, it was fed into the necessary container. If the plasma was to be held for more than 24 hours before the next step, it was fed into sterile 15 gallon polypropylene tanks which were aseptically attached to the second filter.

The filter assemblies used in production were all flushed with 80°C pyrogen-free water prior to use or autoclaving. All filters used were pharmaceutical grade, non-fiber releasing, and manufactured in accordance with current good manufacturing procedures.

6. Despeciation System

The large scale despeciation equipment was designed to reproduce conditions determined to be optimal in small scale experiments. Figure 2 shows the layout of the entire system.

Reaction Tank - During despeciation the plasma was contained in a 30 gallon polypropylene, or 55 gallon polyethylene tank, modified to accommodate a heat exchange coil, mixer, and temperature and pH probes.

Heat Exchange Coil - To heat and cool the plasma during despeciation, a coil of ½ inch I.D. stainless steel tubing, surface area of 5.8 square feet, was immersed in the plasma. Hot (56°C) or cold (2-5°C) water was pumped through this coil to raise or lower the plasma temperature. Temperature probes were mounted in the inlet and outlet of the coil to monitor the efficiency of heat transfer.

Heat Source - To start the reaction, the plasma was warmed to 45°C, as quickly and evenly as possible without exposing it to excessive temperatures. Coil temperature was limited to 56°C during heating. This was done by recirculating water from a tank maintained at 56°C through the coil; 80°C water was periodically added to the tank to replace the heat transferred to the plasma through the coil.

Temperature Monitoring - The temperature was monitored at four points in the system using a digital thermometer with thermistor probes connected through a switch box (Markson Model CR-91 thermometer, Marskin Science, Inc., Phoenix, AZ and Cole Parmer #K-8415-24 thermistors, Cole Parmer Instrument Co., Chicago, IL). The temperature of the heat exchange fluid (water), in the supply tank and at the inlet and outlet of the coil, was monitored and recorded. The temperature of the plasma was also monitored, and was controlled by starting or stopping the flow of water through the coil. Temperatures at all four points were recorded every two

minutes. Because of the mass of the system, heating and cooling curves were very smooth and reproducible. Termination of the despeciation reaction was initiated by replacing the 56°C water in the coil with cold water. Termination of the reaction was completed when the plasma cooled to 20°C.

pH Control - Because the activity of the enzyme used in despeciation, pepsin, is pH dependent, the pH of the plasma was monitored throughout the despeciation procedure. The critical pH for pepsin is 4.0; pepsin activity decreases rapidly above pH 4. In addition, as the despeciation reaction progressed, the pH rose. Therefore, acid was added, periodically, to reduce the pH to 4.

The plasma pH was monitored with two pH meters, Beckman Model 3500 (Beckman Instr.) and Radiometer pH 26 (Radiometer, Copenhagen, Denmark), equipped with combination electrodes and automatic temperature compensation. The Beckman meter was used for small scale runs and so the readings from these were used to determine when to add acid. The Radiometer meter was used to verify proper operation of the Beckman.

7. Pepsin Activation

Pepsin (Calbiochem-Behring, LaJolla, CA lot #202256) was activated immediately prior to use by making a 1% solution of pepsin in 0.01 M HCl/NaOH (HCl from Mallinckrodt, St. Louis, MO; NaOH from Fisher Scientific, Eden Prairie, MN) and mixing for 30 minutes.

8. Despeciation Procedure

The sterile, stabilized plasma was despeciated by digestion with pepsin under controlled conditions of temperature and pH. Prior to despeciation, the plasma was diluted to 20 mg/ml total protein with 56°C normal saline. Sulfuric acid (Allied Chemicals, Morristown, NJ), 0.5 N, was added to the plasma to lower the pH to 4.0. The temperature of the plasma at this point was 30-35°C. Activated pepsin was then added to the plasma, and the heat exchange system, previously described, was used to increase the temperature as rapidly as possible to 45°C. The heat exchange system was designed so that the plasma would reach 45°C within 15 minutes. The temperature of the plasma was maintained at 45°C for approximately 60 minutes. The pH was maintained at 4.0 with acid additions (0.5N sulfuric acid) during this period. At 60 minutes reaction time, the pH was no longer increasing, indicating a complete despeciation reaction. The despeciated plasma was cooled as fast as possible, to 20°C, using the heat exchange equipment. Once the plasma temperature reached 20°C, a saturated tris base solution (trizma base, Sigma Chemical Co., St. Louis, MO) was added to raise the pH to 6.6. The combination of lowering the temperature and raising the pH inactivated the pepsin, preventing further digestion. After the pH was adjusted to 6.6, the plasma was cooled to 10°C or below. To remove insoluble materials, the plasma was then centrifuged at 4°C, 18,000 rpm (32,260 X G), feed

rate 250 ml/min, in a Beckman J2-21 or J2-21M refrigerated centrifuge equipped with a JCF-Z continuous-flow rotor (Beckman Instr.)

9. Batch Anion Exchange

After the despeciation procedure and high-speed centrifugation spin, the inactivated pepsin was removed from the plasma by batch QAE and filtration procedures. A dry anion exchange resin, QAE A-50 sephadex, (Pharmacia, Piscataway, NJ), which binds pepsin, was added to the plasma at a ratio of 1.25 grams per gram of pepsin used in the despeciation. The solution was mixed for one hour, and then allowed to settle overnight. The QAE/pepsin complex was removed by filtration through 29 micron nylon mesh (Nitex, Tetko Inc., Elmsford, NJ). The plasma was then sterile filtered as described earlier. The sterile plasma was stored at 4°C until the next procedure.

10. Concentration and Diafiltration

Protein solutions were concentrated and diafiltered at several points during fractionation, using ultrafiltration (U.F.) systems. For large volumes, a stainless steel, sanitary system (Millipore Pellicon, Millipore, Bedford, MA) with 50 ft.² of Millipore PTGC filters (10,000 MW C.O.) was used. This system was fed with a sanitary lobe pump (Model 10 DO, Waukesha Division, Abex Corp., Waukesha, WI). For small volumes, a Millipore Pellicon with 5 ft.² of filters (10,000 MW C.O.), fed with a peristaltic pump, was used.

Concentration:

After despeciation and batch QAE, the protein solution was concentrated on the large U.F. system to 40-60 mg/ml, in preparation for diafiltration. Following diafiltration, the U.F. system was used to adjust the protein concentration to 60 mg/ml, in preparation for QAE 1. The QAE 1 effluent was then concentrated to 60 mg/ml, prior to QAE 2. The QAE 2 effluent was concentrated to 40-60 mg/ml, for the final diafiltration procedure, and then, finally, to 75 mg/ml for storage.

Diafiltration:

The concentrated, despeciated protein was diafiltered with a pH 6.6 imidazole-acetate buffer (19.4 mM imidazole, 88.8 mM acetate, conductivity 5.85 mS at 21°C) to prepare it for anion exchange chromatography in the same buffer system. Five volumes of buffer were used to ensure 99% buffered exchange; an additional two plus volumes were used to wash out low molecular weight protein fragments. Following QAE 2 and concentration, the protein solution was diafiltered to change the buffer system to glycine-saline at pH 6.8 (0.30 M glycine, 0.154 M NaCl, conductivity 12-15 mS at 21°C) for storage and administration. This final diafiltration also removed any remaining low molecular weight

fragments. Because of the relatively small final volume, the 5 ft.² system was used for this diafiltration procedure to reduce losses due to U.F. system hold-up.

11. Anion Exchange Column Chromatography

Following pepsin digestion and diafiltration, the F(ab')₂ fragments were isolated from the plasma solution by column chromatography. An anion exchange resin, QAE-A50 sephadex (Lot #'s 21887 and 15446, Pharmacia, Piscataway, NJ) was used in 16 liter columns (KS370, Pharmacia). The dry resin was swelled in sterilizing buffer and defined four times before use (the defining procedure is described in the Anion Exchange Chromatography section of the Experimental Fractionation discussion). The resin was then equilibrated with pH 6.6 imidazole acetate buffer (19.4 mM imidazole, 88.8 mM acetate, conductivity of 5.85 mS at 21°C). To prevent microbial contamination and growth, the chromatography system was closed, and buffers were sterilized by filtration before use.

QAE 1:

The digested protein mixture was applied to QAE-packed columns, at a load of approximately 0.5 gm protein per gram dry weight QAE. Maximum load limits were determined experimentally for each QAE lot number; a lower load was used in production to ensure complete separation. Columns were run in parallel, the number used (1-4), dependent upon the amount of protein to be fractionated. Flow rates were maintained at 150 ml/min./stack. To monitor the protein concentration of the column effluent, the effluent was sampled every 4 liters and the absorbance at 280 nm determined with a Gilford Model 2600 spectrophotometer. When the protein concentration of the effluent dropped to 2 mg/ml, collection was complete.

QAE 2:

To remove any remaining contaminants, the effluent from QAE 1 was concentrated, as previously described, and applied to a freshly equilibrated QAE column.

Following QAE 2, the effluent, pure F(ab')₂, was concentrated and diafiltered. It was then high-speed spun at 20,000 RPM (39,900 X G), 21°C, in a Beckman J2-21M centrifuge equipped with a JCF-2 continuous flow rotor, with a feed rate of 250 ml/min. This final centrifugation step removed particulates and aggregates. The product was then delivered to the Quality Control Department for filtration and bottling.

G. Bottling, Storage, Labels, and Shipping

1. Bottling

Sterile bottling of equine antitoxin final products was performed by two different methods, depending on whether the final product resulted from small scale experimental projects intended for analytical purposes, or from large scale production, intended for administration to humans.

Small scale final products were vacuum filtered through 0.22um, 150 ml filter units (#7103, Falcon, Oxnard, CA). While in a laminar flow sterile hood (EdgeGard, Baker Co., Sanford, ME) the product was then removed from the filter unit through the stoppered side arm, using a sterile 60 cc syringe and 15 gauge needle. Observing sterile technique, the product was hand-dispensed, 10 cc per bottle, into steam sterilized 10 cc bottles (#223739, Wheaton, Millville, NJ). Each bottle was sterilely hand stoppered (#546 West Co., Phoenixville, PA or #224124, Wheaton) and hand crimped, with a sterile aluminum seal (West Co.), before being removed from the hood.

Large scale products, intended for human use, were high speed spun (as described earlier) just prior to bottling. The final product was placed in a steam sterilized stainless steel pressure vessel (#C501-0073-14, Alloy Products, Waukesha, WI) and was pressurized to 5-10 psig with medical grade nitrogen (nitrogen, N.F., Ohio Medical Products, Madison, WI). The material was fed through the outlet of the pressure vessel to a prefilter assembly consisting of a stainless steel sanitary housing (#SANNIARP723P, Sealkleen, Pall Trinity Micro Corp., Cortland, NY) and a filter cartridge (ABINRP, Pall Trinity Micro Corp.). The prefilter assembly was previously rinsed with non-pyrogenic water and then cooled to room temperature before use. The filtrate from the prefiltration step was collected in an identical stainless steel pressure vessel. Following pre-filtration, a sample of the final product was taken for protein determination (biuret method).

The material was then pressurized again with medical grade nitrogen, and fed through a sterile 0.2um absolute filter (DFA 3001 NRP, Pall) with a sterile bottling bell attached to the outlet. While wearing caps, gowns, gloves and masks, two technicians dispensed the sterile final product materials into the sterile final product containers. Upon initial bottling, a portion of the material was dispensed into 20 cc bottles (#223742, Wheaton) which were hand stoppered and crimped as described earlier. A sufficient number of bottles were filled with approximately 20 cc per bottle so that the physical and biological characteristics of the lot could be determined using the contents of these vials. The remainder of the lot was "bulk bottled" into sterile, 500 cc bottles (#219759, Wheaton), and closed with sterile black screw caps (#240280, Wheaton). The bulk bottled material was then stored at 4°C while toxin neutralizing activity, and other physical and biological characteristics, were determined.

Following determination of antitoxin neutralizing activity, the product will be final bottled as follows. The adjusted material is high speed spun, before final bottling, and placed in a pressure vessel. Following sterile filtration, using a 0.2 micron absolute filter, the product is collected in a sterile 4 liter Erlenmeyer flask. At approximately the same rate that the product is filtered into the flask, it is drawn from the flask and dispensed into sterile 20 cc bottles by a semi-automated bottling apparatus. This apparatus consists of a Filamatic pump (DAB-16, National Instruments, Baltimore, MD) and a sterile calibrated stainless steel syringe (FUS-16, National Instr.) with a bottling bell attached. Two technicians, wearing sterile caps, gowns, masks and gloves, perform the bottling procedure under the laminar flow hood observing sterile technique. Each bottle is filled with 20 cc of final product and is hand stoppered and crimped immediately after filling.

2. Storage

Small scale experimental final products were stored at either 4°C or -20°C. The large scale, "bulk-bottled" final products were stored at 4°C until the final bottling (at the adjusted IU/ml). After final bottling and labelling, the products will be stored at -20°C.

3. Labels

In accordance with CFR610.60, each final bottle will be labelled with a custom designed label describing the name of the product, name and address of the manufacturer, lot number, potency (antibody titers, IU/ml, to botulinum toxins A through G), and the recommended individual dose.

4. Shipping

The final products and fractionation samples were sent to Fort Detrick, Frederick, Maryland via Emery Express overnight service. To maintain the temperature of the products at 4°C during shipping, experimental and testing bottles were packed in styrofoam boxes containing frozen Kool-its.

The final products prepared for human use will be placed into custom made, individual boxes, bearing the same label affixed to the bottle contained. These bottles will be shipped frozen, packed in insulated styrofoam boxes containing sufficient dry ice to maintain keep the products in a frozen state.

H. Quality Control

1. Sterility, CFR610.12

In accordance with CFR610.12, all products intended for human use were sterility tested before being released for human use. Several (2-20) randomly selected bottles from each lot were submitted to Economics Laboratory, Inc. (Mendota Hts., MN) for sterility testing. As of June, 1983, until April, 1986, sterility samples were tested by Medtronics, Inc. (Coon Rapids, MN). Currently, sterility testing is performed by the Microbiology Department at the ALG Program.

To perform the sterility test, the contents of each bottle were inoculated into thioglycollate medium and soybean casein digest medium, and incubated for 14 days at 30-32°C and 20-25°C, respectively. These test mediums were observed for growth on days three, four, seven, eight and fourteen. Following completion of the test, the testing facility submitted a written report of the final product sterility testing results. For the material to pass the sterility testing, Regulation CFR610.12 requires that no growth appear in any of the cultures.

2. Pyrogen Test, CFR610.13b

All final products intended for human use were tested for the absence of pyrogenic substances, in accordance with CFR610.13b as follows. Three to four previously conditioned New Zealand White rabbits (Dutchland Lab., Denver, PA, 3-5 lb., females) were injected intravenously, via an ear vein, with the final product material. Each rabbit received a dose of 1 ml of product per kilogram of body weight; their temperatures were monitored for three hours. Prior to injection, a normal baseline temperature was established and recorded. For the products to pass the pyrogen test, no rabbit's post injection temperature could deviate 0.6°C or greater from its baseline temperature.

3. General Safety Test, CFR610.11

The general toxicity of final products intended for human use was tested, in accordance with CFR610.11 as follows. In independent tests, the final products were warmed to 37°C and injected intraperitoneally into two guinea pigs (Gopher State Caviary, White Bear Lake, MN, 400 grams each) and two mice (Bio Labs, St. Paul, MN, <22 grams each). Each guinea pig was injected with 5 cc of product, each mouse with 0.5 cc. Each working day, over a period of seven days, the animals were observed for overt health. On day seven, they were weighed, and their weights recorded. To pass the requirements of the General Safety Test, all the animals must remain overtly healthy and lose no weight during the test interval.

4. Immunoelectrophoresis (IEP) of Final Products

Purpose:

Final products were tested for purity and electrophoretic mobility by immunoelectrophoresis.

Procedure:

Commercially available IEP kits and antisera were used to test the final product. A 3 μ l volume of sample was placed into a well in a pre-cut IEP film gel (1% agarose). Samples were electrophoresed for 100 minutes at 80 volts to separate the proteins (due to their electrophoretic mobility). Anti-whole human serum or anti-human IgG was then added to the adjacent trough(s). The gel film incubated overnight at room temperature in a humidified chamber. During incubation the sample and antisera diffused through the agar, formed a complex, and precipitated. Each protein present in the sample resulted in a unique and characteristic precipitate. IgG precipitated to the left of the sample well (cathodal migration). Most other plasma proteins precipitated to the right of the well (anodal migration).

Purity was determined by staining the gel to enhance and visualize the protein precipitates. The gel was repeatedly washed in saline to remove unbound proteins, rinsed in water, and dehydrated. It was then stained using 5% Coomassie Blue Stain: 5 gm Coomassie Blue in destain solution (10% glacial acetic acid, 45% ethanol, 45% distilled H₂O). The film was destained and dried. A permanent record was made by storing the stained film in a plastic packet; photographic copies were also kept.

5. Isoelectric Focusing (IEF) of Final Products

Purpose:

An IEF gel was used to determine the isoelectric point (pI) range of the final product. Trace contaminants of non-IgG proteins, which may not be demonstrated by High Performance Liquid Chromatography (HPLC) or IEP, can be detected by this method.

Procedure:

A precast IEF gel, pH 3.5-pH 9.5, was electrofocused with the final product and pI standards. The final product was applied at 10 mg/ml, 30 mg/ml, and full strength. The proteins migrated through the gel. When the proteins reached their pI's, the migration stopped. Following electrofocusing, the gel was fixed, stained, and destained. The gels were analyzed to determine final product purity, and a permanent photographic record was made.

III. Results

A. Immunization of Horses

First Flight was immunized 49 times with botulinal toxoids and/or toxins; sixteen immunizations were administered since his arrival to Minnesota. Abe received thirteen immunizations prior to his arrival to Minnesota, and two in Minnesota. Tables I and II summarize the immunization schedules, and toxoid or toxin types injected while at Fort Detrick and at the University of Minnesota.

B. Plasmapheresis

First Flight was plasmapheresed 121 times since arriving in Minnesota. The plasmapheresis dates, and plasma protein concentrations are shown in Table III for First Flight bleeds 1-121. This horse tolerated this extended schedule quite well, due to both his cooperative disposition and the expertise with which the bleeding and reinfusion were performed by the ALG veterinary student bleed crew. These experienced workers could bleed horses with a minimum amount of trauma to the horse or damage to the jugular vein, which is essential for long-term repeated plasmapheresis.

Antitoxin neutralizing activity (IU/ml) for First Flight bleed pools 1-14 (bleeds 1-57) are shown on the bar graph, Figure 3. Antitoxin titers for bleeds 58-121 have not been completed yet. Except for initially high antitoxin A titers, the activity against Type A and Type B toxins was maintained at consistent levels throughout the bleed schedule. Thus, our plasmapheresis schedule and technique did not diminish the antitoxin titers, as one might expect such a seemingly stressful procedure to do. On the contrary, plasmapheresis has been shown to stimulate or enhance circulating antibody titers (11).

Although the antitoxin titers of First Flight bleeds 58-113 have not been completed, we expect that these titers will be as high or higher than bleeds 1-57 due to the generally shorter duration between immunizations during this period (see Table I).

Abe was plasmapheresed sixteen times. Plasmapheresis dates, and plasma protein concentrations for Abe, bleeds 1-16 are shown on Table IV. The antitoxin titers have not been determined yet.

IgG(T) Levels:

The concentration of IgG(T) in First Flight and Abe plasma samples was determined by RID and is reported on Tables III and IV, respectively. This "T" immunoglobulin class has been shown to possess significant neutralizing antitoxin activity. Figures 4 (a, b and c) and 5 summarize the immunization and bleed schedules, total protein concentrations, IgG, and IgG(T) levels of the plasma collected from First Flight B(1-113) and Abe B(1-16).

C. Plasma Storage

An inventory of plasma obtained by plasmapheresis of First Flight and Abe was maintained at -20°C . Plasma was removed from inventory for digestion experiments, special lot production, bleed pool A preparation and for the production of FFA1 and FFA2.

D. Experimental Fractionation

1. Goals of Fractionation Experiments

The general goal of the fractionation experiments was to determine a fractionation scheme which produced a material that is both safe when administered intravenously, and efficacious in the treatment of botulism intoxication by types A, B, C, D, E, F, and G botulinal toxins. The fractionation scheme was to include a method of eliminating the most immunogenic portion of the immunoglobulins, the pFc' portion, while still maintaining specific toxin neutralizing ability. Thus, the optimal method of despeciation, using pepsin as the proteolytic enzyme, was determined by studying the effect(s) of the following variables during the despeciation of equine plasma: pH, temperature, protein concentration, pepsin concentration, incubation time, and ionic strength.

2. Historical Overview

Despeciation with Proteolytic Enzymes:

The use of proteolytic enzymes to purify complex protein mixtures by digestion is not new. Typically, the digestion involves long incubations with pepsin (several days at 37°C), and the presence of an antibacterial agent such as phenol. This technique has also been used to despeciate equine plasma containing antibodies specific for tetanus and botulinal toxins. The purpose of the despeciation is to reduce the immunogenicity of horse plasma by the elimination of the pFc' portion of the immunoglobulins, leaving only the F(ab')_2 portion. No one, however, has produced an effective equine F(ab')_2 preparation that is free of non-immunoglobulin proteins, aggregates, and residual intact immunoglobulins.

The Japanese Method:

Advances have been made in the preparation of despeciated equine antitoxins, most notably by the Japanese. The Japanese method reduces the time needed for digestion, from 2 days to 30 minutes, by increasing the reaction temperature, and adding ammonium sulfate to protect the immunoglobulins from denaturing at high temperatures (12). The final antitoxin products produced in our laboratory by this despeciation methodology contained approximately 60-70% F(ab')_2 fragments, and 30-40% intact IgG. In addition, there were relatively large quantities of aggregated proteins which we considered unacceptable. A comparison of the

various molecular species in commercial preparations is illustrated in Figure 6. The Japanese and Connaught equine botulin antitoxin products have been analyzed on a Biogel molecular column, where the various protein components are separated on the basis of molecular size. The Connaught preparation is a product of the classical pepsin despeciation (temperature 37°C, despeciation over 5-7 days). None of these procedures has resulted in an antitoxin which is pure $F(ab')_2$ and free of allergic reactions upon human administration.

Despeciating IgG:

Instead of despeciating equine plasma, and then purifying the resulting $F(ab')_2$ fragments, pure equine $F(ab')_2$ could be obtained by starting with pure equine gamma globulin, and then digesting these proteins with pepsin. This method, which has not been tried on a commercial scale, was not chosen for large scale production for the reason following. The method of equine immunoglobulin purification used in this laboratory is anion exchange chromatography. Horse plasma contains, in addition to IgG, another immunologically important class of immunoglobulin. IgG(T), therefore, does not elute with IgG during anion exchange chromatography. As a result, the product does not contain despeciated IgG(T). Upon digestion of equine plasma with pepsin, however, the IgG(T) $F(ab')_2$ fragments formed are more basic than the whole molecule, and can then be eluted during anion exchange chromatography with the IgG fragments. This is also true for acidic IgG. Thus, despeciation prior to anion exchange chromatography was expected to result in higher yields of toxin neutralizing activity compared to despeciation after chromatographic isolation of the gamma globulins.

Despeciation of Equine Anti-Ovine IgG:

To illustrate this point, plasma from a horse immunized with ovine IgG was fractionated. The plasma contained precipitating antibodies to ovine IgG. Fractionation of the IgG from plasma resulted in large quantities of monomeric equine IgG containing almost no precipitating antibody against ovine IgG. Preparative focusing of the plasma showed that the anti-ovine IgG activity was located in an acidic pH range corresponding to unrecoverable IgG and IgG(T). Pepsin treatment of the plasma prior to anion exchange chromatography, however, resulted in a pure $F(ab')_2$ final product containing a high yield of anti-ovine IgG precipitating antibodies. Thus, the removal of the pFc' portion of these antibodies resulted in a change in net charge, and the more active fragments, now more basic, were eluted during anion exchange chromatography.

3. Experimental Fractionation

Despecciation of IgG:

The first fractionation experiments involved purifying equine IgG and IgG(T) prior to despecciation. Equine plasma was first stabilized by clotting or adding silicon dioxide (SiO₂). The stabilized plasma was then applied to an anion exchange column. By eluting the bound protein at a lower pH than the running buffer, a mixture containing IgG, IgG(T), and non-immunoglobulin proteins was collected. Pepsin was then added to despecciate this material. The product resulting, however, contained only aggregates and intact IgG. Modifications of the despecciation procedure, such as changing the pepsin concentration, pH, temperature, or reaction time, failed to produce an acceptable yield of F(ab')₂ fragments.

Despecciation of Horse Plasma - Japanese Method:

Later experiments all involved the despecciation of horse plasma (SiO₂ stabilized or "as is") prior to any purification procedure. The first of these experiments duplicated the Japanese method of despecciation (12). This method involved a 30 minute digestion of plasma at 58°C, pH 4.6, in the presence of 26% SAS. The products resulting, even after chromatography, contained aggregates, non-immunoglobulin proteins, and intact IgG, as did a sample of the Japanese produced antitoxin. An increase in pepsin concentration, from 3% to 9% did not increase the F(ab')₂ content above 70%. Higher pepsin concentrations, rather than reducing the concentration of intact IgG, merely resulted in decreased yields of antitoxin.

The Minnesota ALG Method:

Inclusion of ammonium sulfate in the digestion mixture, for protection of the protein from denaturation during despecciation at a high temperature, was questioned. Ammonium sulfate has been used for many years to isolate IgG from plasma by a two step salting-out procedure. Its presence in the digestion mixture, far from being beneficial, could actually inhibit pepsin digestion by preventing peptide chain unfolding, thereby making the IgG less susceptible to pepsin proteolysis. In effect, the SAS in the Japanese procedure was counteracting the purpose of the high temperature, unfolding the IgG peptide chains. Addition of ammonium sulfate to the digestion mixture would also inhibit pepsin digestion by increasing ionic strength; pepsin activity is reduced at high ionic strength. To test this idea, the Japanese conditions without ammonium sulfate were repeated on both SiO₂-stabilized horse plasma, and horse plasma "as is", and using various concentrations of pepsin. The results indicated that both despecciation without ammonium sulfate, and SiO₂ treatment of the plasma prior to despecciation were beneficial. Despecciation of non-stabilized plasma led to difficulties during anion exchange chromatography (back pressure, flow rate decreases), resulting in

decreased antitoxin yields. In addition, the final product contained both $F(ab')_2$ and intact IgG. To eliminate the remaining intact IgG, the variables present during despeciation were studied. These variables included: temperature, time, and pH of the digestion reaction, protein concentration, pepsin concentration, and ionic strength of the digestion mixture. Critical variables were identified and then modified until the optimum conditions resulting in a product with acceptable purity, specific activity, and yield were found. We found two significant factors that worked synergistically--the first, temperature, and the second, pH. When the pH was lower than 4.6 and the temperature less than 50°C, digestion proceeded to completion in less than one hour, and the final product was essentially over 99% $F(ab')_2$ equine immunoglobulin.

Pepsin Concentration:

Pepsin concentration was one of the first variables studied. Experiments showed that increasing the pepsin concentration as much as 9-fold resulted in only slightly less intact IgG in the final product. Thus, high pepsin concentration was not especially advantageous, and the pepsin concentration selected for large scale despeciation was 3%. Less pepsin made the procedure more cost effective.

Digestion Time:

Allowing the digestion to proceed for three hours instead of one hour resulted in nearly identical final products. Indeed, the vast majority of proteolytic activity occurred during the first 15 minutes of digestion. The digestion time chosen for large scale despeciation was 60 minutes.

Ionic Strength:

Ionic strength of the digestion mixture was also varied, and the ionic strength of normal saline, 0.154, was found to be optimal. At extremely low ionic strength, some proteins become insoluble. At higher ionic strength, the proteolytic activity of pepsin is reduced.

Protein Concentration:

Experiments which varied the protein concentration demonstrated that protein concentrations varying from 10 mg/ml to 50 mg/ml yielded similar products following pepsin digestion. A concentration of 20 mg/ml was chosen for large scale despeciation.

pH and Temperature:

The pH and temperature during digestion proved to be the most critical variables. Maximal activity of pepsin occurs at pH 1.75, with activity decreasing as pH increases. Pepsin is inactive at a

pH above 5.5. A plasma pH approaching 1.75 would result in more complete digestion, but protein denatures at low pH. Therefore, a compromise pH, between 1.75 and 5.5, was chosen to ensure high enzyme activity without denaturing the protein; pH 4.0 was this compromise. In addition, it was discovered that the plasma pH increased during the digestion period, especially during the initial 15 minutes, due to released digestive products. Maintaining the plasma pH at 4.0, by adding acid as needed, allowed lowering of the digestion temperature from 58°C to 45°C. This made large scale production easier.

E. Fractionation Production

1. Plasma Pools

Containers of plasma from FF Bleed Pool A were selected from inventory for production of FFA1-3-45-4x1 (FFA1) and FFA2-3-45-4x1 (FFA2), and thawed to 4°C. Fourteen containers with a total inventory volume of 52.4 liters yielded an FFA1 pool volume of 51.9 liters at 51.2 mg/ml; total protein was 2657 gm. The FFA2 plasma pool consisted of 25 containers with 95 liters of inventory volume, yielding 4874 gm of protein (51.3 mg/ml). The thawed plasma for FFA1 was pooled in a sterile 15 gallon tank. The plasma for FFA2 was pooled in a sanitized 30 gallon tank. Each pool was mixed and sampled before stabilization.

2. Stabilization of Plasma

Silicon dioxide was added to the plasma at a ratio of 15 gm SiO_2 /liter of plasma. After removal of the SiO_2 , by centrifugation, and sterile filtration, the plasma was clear. Plasma volumes decreased from 51.9 liters to 47 liters for FFA1 and from 95 liters to 90.7 liters for FFA2 due to adsorption by SiO_2 and small handling losses during the centrifugation and filtration procedures. The gram protein yield through these steps was 2035 gm (76.6%) for FFA1 and 3699 gm (75.9%) for FFA2.

3. Preparation for Despeciation by Pepsin

The plasma was warmed to ambient temperature (approximately 20°C) either during the final SiO_2 removal spin or following post- SiO_2 sterile filtration. A normal saline (0.9%) solution at 56°C was used to both dilute the plasma, from approximately 42 mg/ml to 20.0 mg/ml, and warm the plasma to 33°C. This initial warming reduced the time required to heat the plasma to 45°C following pepsin addition. The pH of the plasma was adjusted to 4.00 ± 0.01 by adding 4.50 liters of 0.5 N H_2SO_4 to FFA1, and 7.77 liters to FFA2. All acid and base solutions were added slowly, while mixing the plasma rapidly, to prevent local denaturation. Mixing was interrupted periodically to allow a more stable pH reading.

Concurrent with the above procedures, pepsin was activated in a 20°C solution of 0.01 N HCl/NaOH , pH 3.00 ± 0.01 . For FFA1, 61.4 gm of pepsin were added to 6.14 liters to make a 10 mg/ml solution. For

FFA2, 110.97 gm in 11.1 liters were used. These pepsin quantities represented 3% of the total protein in the plasma just prior to despeciation. The activation time allowed for the pepsin used in FFA1 was 37 minutes, due to delays in the pH adjustment of the plasma. For the pepsin used in FFA2, the activation time allowed was 30 minutes.

4. Despeciation by Pepsin

The despeciation period extended from the time at which the pepsin was added to the plasma (T=0) to the time when the plasma was adjusted to pH 6.6, thus stopping the action of the pepsin. Figures 7 and 8 show the temperature and pH curves as well as the times and volumes of acid and base additions for FFA1 and FFA2, respectively.

For FFA1, the time elapsed during the heating cycle (from 33.2°C to 45.0°C) was 9 minutes. The temperature was held at 45°C+0.3°C for 65 minutes. The pH increased to 4.14 at T=10 minutes and was reduced to 3.97 with the addition of 480 ml of 0.5 N H₂SO₄. A small amount of acid (30 ml) was added at T=42 min., to reduce a small increase in pH. At T=74 min. the cooling cycle was started. By T=84 (10 minutes into the cooling cycle), the temperature had dropped to 21.9°C. The pH was then adjusted to 6.6 using 1.1 liters of saturated Tris base, 20°C. These conditions of lower temperature and higher pH were expected to prevent any further enzyme activity. The plasma was then cooled to 10.9°C.

The same equipment used in the despeciation of FFA1 was used to despeciate FFA2. Consequently, the significant increase in starting plasma volume for FFA2 (51.9 liters for FFA1 versus 95 liters for FFA2) resulted in a longer heating cycle (from 33.0°C to 45°C) of 15 min. The greater system mass also resulted in a smoother rise in temperature and greater stability at 45°C (+0.1, -0.2°C). This temperature was maintained for 60 minutes. It was previously decided that the pH would be adjusted more frequently during digestion of FFA2 than was done for FFA1. The first addition of acid was at T=5 minutes; the pH was 4.10 and was reduced to 4.02 with 0.82 liters of 0.5 N H₂SO₄. Three more additions were made at T=15 min., T=38 min. and T=58 min., adding a total of 0.765 liters of acid. The pH was thus maintained at 4.00+0.04, -0.00. Ideally, a system of constant adjustment, such as an automatic titrator, would be used. The cooling cycle was started at T=75 min. By T=94 min. (19 min. into cooling cycle), the temperature had reached 20.1°C. The cooling cycle was interrupted to allow careful adjustment of the pH to 6.60 by addition of 2.03 liters of saturated Tris base, 20°C. The cooling cycle was continued after this 4 minute break until the plasma temperature reached 10.1°C at T=122 min.

Samples were taken during the despeciation procedures for analysis by HPLC (see Experimental Testing section for description of procedure). Figures 9 (FFA1) and 10 (FFA2) show graphs of molecular weight distribution in these samples over the course of the

despeciation procedure, as well as after concentration and diafiltration and QAE procedures. These last two graphs will be discussed later.

The first graph in each figure shows the molecular weight distribution at T=0 min., before any significant enzyme action has occurred. The intact IgG appears between 145,000 and 155,000 molecular weight. The next two graphs, at T=5 min. and T=15 min., show changes due to enzyme action. The intact IgG peak shifts towards the lower molecular weight of 90-98,000 and then appears as a decreasing shoulder on the F(ab')₂ peak at 98,000. At the same time the peptide fragments in the low molecular weight region (0-40,000) are rapidly increasing. At T=45 and 55 min. the 98,000 peak is nearly symmetrical, and the quantity in the low molecular weight region is quite large. The high molecular weight peak, at the void volume of the column, is reduced rapidly during the early part of the sequence.

5. Batch Anion Exchange

After despeciation, the plasma was high speed spun, QAE was added to remove the inactivated pepsin, and the plasma was sterile filtered. The yield after these procedures was 1799 grams of protein (67.7%) in 107.4 liters for FFA1, and 3185 grams (65.3%) in 195.5 liters for FFA2. Losses for these procedures were due to handling, adsorption of protein to QAE (largely non-F(ab')₂), and removal of aggregated protein.

6. Preparation for Anion Exchange Chromatography (QAE); Concentration and Diafiltration #1

Following the sterile filtration procedure, post-Batch QAE, the protein mixture was concentrated to approximately 50 mg/ml and diafiltered with imidazole-acetate buffer, pH 6.6, conductivity 5.85 mS at 21°C. Seven volumes of this buffer were passed through the plasma to wash out other salts and low molecular weight peptide fragments. The membrane filter used in concentration and diafiltration has a nominal molecular weight cutoff of 10,000. As shown in Figure 9 and 10, and T=55 min. samples for FFA1 and FFA2, respectively, were below this molecular weight. The 5th graph in each of these figures, entitled "Post 10,000 MW Membrane", shows a significant decrease in this range. The percent yield for the diafiltration procedure was approximately 32% for FFA1, and 44.5% for FFA2. The wash-out of low molecular weight fragments was apparently less efficient for FFA2 than for FFA1. That this was the case rather than some other type of loss was confirmed by unexpected losses for FFA2 during the pre-QAE 2 and final concentrations and diafiltrations; these losses did not occur for FFA1. The estimated total percent lost attributable to 10,000 MW filtration procedures for FFA2 (38%) was similar to the percent loss for the first such procedure in FFA1 (32%). This loss did not adversely affect the purity of the final product.

The diafiltered plasma was high-speed spun and warmed to 20°C prior to the QAE1 procedure. The yield for this step (including con-

centration and diafiltration and the pre-QAE high-speed spin) was 574 grams for FFA1 and 1416 gm for FFA2.

7. QAE1

For FFA1, 9.8 liters of diafiltered plasma at a concentration of 58.6 mg/ml (574 gm) was applied to 2632 gm of QAE-A50 sephadex in 4 parallel columns. The protein load for this run was 0.22 gm protein per gram of dry weight QAE. This especially low load was used to ensure a pure product. A total of 56 liters of eluant was collected yielding 282 gm, or 49.1% of the protein applied. This protein was concentrated and diafiltered with a pH 6.8 glycine-saline solution (0.3 M glycine, 0.154 M NaCl, 12-15 mS conductivity at 21°C). It was then high-speed spun, sterile filtered and bulk bottled. When a quality control check on the pH of the equilibration buffer showed a pH of 5.8 instead of 6.6, a second QAE procedure (QAE2) was scheduled. It was discovered that the equilibration buffer for QAE1 was made and adjusted using the pH probe from the despeciation procedure. The probe was excessively slow because of exposure to rapidly changing temperatures and Tris base. During production of FFA2, the pH probes used in despeciation were cleaned and carefully tested before use in other solutions.

Typically, the maximum load for a QAE1 procedure is approximately 0.5 gram protein per gram of dry weight QAE. FFA2 was sized to use this maximum load, adding a QAE2 procedure to remove any breakthrough contaminants. The actual load for FFA2 was 1416 gram protein at 59.5 mg/ml (23.8 liters), or 0.54 gram protein per gram QAE. The yield for this run was 100 liters of eluant containing 711 gm of F(ab')₂, or 50.2% of the total protein applied. This material was concentrated and diafiltrated with pH 6.6 imidazole-acetate buffer in preparation for QAE2. A larger than expected protein loss (106 gm) occurred in this step due to the wash-out of some remaining low molecular weight protein, as previously discussed.

8. QAE2

A second QAE procedure is used when there is reason to believe the first QAE procedure did not yield a completely pure product, due to unexpected problems in fractionation or use of a high load to maximize yield and QAE use. Because of the low equilibration pH for QAE1 during production of FFA1, the bulk bottled material was pooled and diafiltered with pH 6.6 imidazole-acetate buffer in preparation for QAE2. A volume of 4.01 liters containing 231 gm at 57.7 mg/ml was applied to 658 gm of QAE (one column) for a load of 0.35. The yield was 22 liters containing 222 gm, or 96.1% of the applied protein. This protein was concentrated and diafiltrated with pH 6.8 glycine-saline solution, and then high-speed spun and delivered to Quality Control for sterile filtration and bulk bottling. The total quantity at this stage was 2.5 liters containing 204 gm. This was an overall yield of 7.7% from the starting total protein, or 3.93 gm/liter of plasma.

For FFA2, 12.0 liters containing 605 gm was applied to 531 gm QAE, for a load of 1.14. This yielded 38 liters of eluant containing 600 gm of F(ab')₂, or 99.2% of the applied protein. This protein solution was then diafiltered with 7 volumes of pH 6.8 glycine-saline solution. A loss of 110 gm occurred during this step, due to the wash-out of low molecular weight peptide fragments, as previously discussed.

The yield for FFA2 was 6.60 liters containing 490 gm of F(ab')₂. This represents a yield of 10.1% of the starting protein total and a gram/liter yield of 5.15. This is higher than the 3.93 gm/liter yield of FFA1 because FFA1 production included several additional handling steps related to the unexpected QAE2 procedure.

To achieve a single uniform product and to reduce the losses incurred by separate quality control procedures, the FFA1 product remaining at the time of FFA2 production was added to FFA2 just prior to the final high-speed spin. This quantity amounted to 94 gm, bringing the total quantity of F(ab')₂ delivered for sterile filtration and bulk bottling to 7.75 liters at 75.35 mg/ml, or 584 gm.

F. Bottling, Storage, Labels and Shipping

1. Bottling and Storage

The final products were initially "bulk" bottled in 500 cc containers. After toxin titers were determined, the materials were then to be rebottled in 20 cc bottles.

FFA1 was "bulk" bottled for the first time on December 23, 1982. Shortly after bottling it was discovered that the QAE elution buffer used to purify this lot was not at the optimum pH. FFA1 was then scheduled for a second QAE procedure (see Fractionation Production Results). For this reason no samples of the December 23rd bottling were submitted for sterility or purity testing.

The material was unbottled, passed over a second QAE stack, and rebottled on January 7, 1983. The prebottle, post high-speed spin volume was 2.51 liters. The lot was sterile filtered into forty-two 20 cc bottles and four 500 cc bottles. Four of the 20 cc bottles were used for sterility and testing purposes. Twenty bottles were filled and shipped to Major Crumrine for the purpose of establishing an inventory of antitoxin at Fort Detrick that could be used for emergency treatment of personnel in the event of accidental toxin poisoning.

FFA1-3-45-4X1 final product "bulk" material, approximately 94 grams (1250 cc), was stored at 4°C until mid April, 1983. At that time it was decided to pool this material with the FFA2-3-45-4X1 final product, prebottle material. Had the 1250 cc of FFA1-3-45-4X1 product been rebottled in 20 cc vials as planned, over 30% of the final product vials would have had to be submitted for sterility testing, in

conformance with CFR610.12 (20 bottles per lot are tested). This would have been wasteful, yielding only approximately 35 bottles available for patient use.

FFA2-3-45-4X1 (including 1250 cc of FFA1-3-45-4X1) was bottled on April 21, 1983. The total prebottle volume was 7.75 liters, yielding 17 X 500 cc bottles ("bulk" material) and 9 X 20 cc bottles for testing purposes. Two bottles of FFA2 were submitted for sterility testing. The "bulk" material is being stored at 4°C until the antitoxin titers have been determined. Appendices 2, FFA1-3-45-4X1, and 3, FFA2-3-45-4X1, summarize the bottling characteristics and yields of each final product.

Following determination of antitoxin neutralizing activity, the bulk material will be high speed centrifuged, and sterilely bottled into 20 cc bottles. Twenty bottles will be submitted to the ALG Microbiology Department for sterility testing, and 10 bottles retained for testing purposes.

2. Labels

A proposed label (Figure 11) has been sent to Fort Detrick for approval. When the label has been approved, the label will be printed and affixed to the 20 cc bottles. The product can then be shipped to Fort Detrick.

3. Shipping

Samples of each lot were shipped to Fort Detrick for the purpose of determining the neutralizing activity of these preparations. As discussed earlier, 20 bottles of FFA1-3-45-4X1 were shipped to Fort Detrick for emergency use. The shipment dates and quantities were as follows:

FFA1-3-45-4X1	15 X 20 cc bottles	January 12, 1983
FFA1-3-45-4X1	20 X 20 cc bottles	January 24, 1983
FFA2-3-45-4X1	5 X 20 cc bottles	April 27, 1983

G. Final Product Testing

1. Sterility

Two 20 cc bottles (the first and last filled), each from FFA1-3-45-4X1 (1-7-83) and FFA2-3-45-4X1 (4-21-83), were submitted to Economics Laboratory, Inc. (Mendota Hts., MN) for sterility testing. These bottles vials were tested to be certain that each lot was sterile during 4°C storage. Written reports of sterility testing were submitted by the testing facility. Both lots were determined to be sterile (see Appendices 4 and 5). After final bottling in 20 cc bottles, for human use, 20 bottles will be selected at random to represent all stages of bottling. These will be numbered 1-20 and submitted to the Microbiology Department for sterility testing, in accordance with CFR610.12.

2. Pyrogenicity

Final product lots FFA1-3-45-4X1 and FFA2-3-45-4X1 were tested for the presence of pyrogens, in accordance with CFR610.13b. No rabbit's temperature increased 0.6°C or higher from its baseline temperature, as indicated by the dotted line on Figures 12 and 13; therefore, these lots passed the requirements of the test for the absence of pyrogenic substances. Appendices 6 and 7 and Figures 12 and 13 summarize the pyrogen testing results.

3. General Safety

FFA1-3-45-4X1 and FFA2-3-45-4X1 were tested for general toxicity in accordance with CFR610.11. All animals gained weight and remained healthy throughout the 7 day test period; therefore, these lots passed the requirements of the general safety test. The results of these tests are shown in Appendices 8, FFA1-3-45-4X1, and 9, FFA2-3-45-4X1.

4. Immunoelectrophoresis of Final Products

FFA1-3-45-4X1 and FFA2-3-45-4X1 final product preparations were tested for purity and electrophoretic mobility. The samples were electrophoresed and then precipitated with rabbit anti-whole equine serum or rabbit anti-equine IgG. Figures 14 and 15 illustrate the precipitin bands that developed. There were three precipitin bands in both samples run against anti-whole equine. The two major bands are characteristic of despeciated IgG. The third barely visible precipitin, more anodally located, may be trace amounts of transferrin. Despeciation affects many of the plasma proteins, altering their normal electrophoretic mobility and making it difficult to definitively identify them.

The final products precipitated with anti-equine IgG elicited the same double band precipitate shown against anti-whole serum, but did not show the third trace precipitin band.

5. Isoelectric Focusing

FFA1-3-45-4x1 and FFA2-3-45-4x1 final products were analyzed by IEP to evaluate purity. The FFA1-3-45-4x1 displays the typical heterogenous banding in the pH range 9.30 to 5.6 for despeciated gammaglobulin. A single contaminant band at approximately pH 5.3 becomes visible in the 25 mg/ml dilution and also at 50 mg/ml. The FFA2-3-45-4x1 similarly shows the heterogeneous banding and the single contaminant. However, it is also visible in the 10 mg/ml dilution which indicates a slightly higher concentration in this preparation.

IV. References

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BLEED POOL A COMPOSITION

<u>BLEED #</u>	<u>CONTAINER DESIGNATION</u>	<u>VOLUME LITERS</u>	<u>BLEED #</u>	<u>CONTAINER D DESIGNATION</u>	<u>VOLUME LITERS</u>
1	A,B,C,D	2.8	31	A,B	4.8
2	B	1.5	32	A,B	7.3
3	B	2.3	33	A,B	7.4
4	B	3.1	34	A,B	6.75
1-4 pool	C,D	2.05	35	A,B	7.1
5	A,B	6.8	36	A,B	6.65
6	A,B	6.3	37	A,B	7.3
7	A,B	5.5	38	A,B	6.25
8	A,B	6.1	39	A,B	5.95
9	A,B	7.43	40	A,B	6.1
10	B	3.56	41	A,B	6.0
11	A,B	5.8	42	A,B	7.15
12	A,B	6.68	43	A,B	6.6
13	A,B	6.3	44	A,B	7.0
14	B	2.55	45	A,B	7.2
15	A,B	5.7	50	A,B	7.1
16	A,B	5.35	51	A,B	6.0
17	A,B	7.6	52	A,B	6.45
18	A,B	3.42	53	A,B	6.1
19	A,B	6.14	54	A,B	6.7
20	A,B	7.0	55	A,B	6.57
21	A	2.4	56	A,B	6.6
22	B	3.7	57	A,B	6.3
23	A,B	5.34	58	A,B,C	7.05
24	A,B	6.5	59	A,B	6.9
25	A,B	7.05	60	A,B,C	6.75
26	B	3.8	61	A,B	6.9
27	A,B	6.47	62	A,B,C	7.07
28	A,B	7.0	63	A,B,C	6.53
29	A,B	7.0	64	A,B,C	7.25
30	B	2.7	65	A,B	5.9

BLEED POOL A COMPOSITION

<u>BLEED #</u>	<u>CONTAINER DESIGNATION</u>	<u>VOLUME LITERS</u>
66	A,B	7.5
67	A,B,C	7.1
68	A,B,C	7.05
69	A,B,C	6.85
70	A,B	6.8
71	A,B	6.75
72	A,B,C	6.75
73	A,B,C	7.05
34-57 pool	A-G	<u>6.0</u>
TOTAL VOLUME*		423.51 liters

*"C" containers and "34-57" pool were sample volumes and were not included in the general inventory.

Final Product Record Sheet

Lot # FFAI-3-45-4X1Bottled 12-23-82

Re-OAE'd
Re-bottled 07/1-7-83

	date, initials	Results
PHSS volume	<u>RF 12-23-82</u>	<u>3.24 L</u>
Conductivity	<u>12-23-82 RF</u>	<u>10.5 @ 21°</u>
pH	<u>12-23-82 RF</u>	<u>6.78</u>
Protein	<u>12-23-82 MF</u>	<u>77.8 mg/ml g/vial</u>
Date Bottled	<u>12-23-82 JPLW</u>	Vials - Total: <u>20,120 L</u> To Ship: <u>5,700</u>
		Grams - Total: _____ To Ship: _____
Assay	date, initials	
Cytotoxicity	<u>not done</u>	10 mg/ml: _____ As Is: _____
Henagglutination	<u>not done</u>	10 mg/ml: _____ As Is: _____
Pyrogen (610.13b) ✓	<u>12-29-82 JP</u>	pass: <u>X</u> fail: _____ Av. High Dev.: _____
Platelet Aggregation ✓	<u>not done</u>	# seconds at 10 mg/ml _____
Safety (610.11) ✓	<u>not done</u>	pass: _____ fail: _____
EAA (610.40)	<u>not done</u>	Antigen: (+) or (-) _____
IEP ✓	<u>12-29-82 JV</u>	Antibody: (+) or (-) _____
		mg/L
Sterility (610.12) ✓	<u>ECI 12-23-82</u>	Sterile: _____ Nonsterile: _____
EGG ? ✓		
Mixed Agglutination	<u>not done</u>	1: _____ at 10 mg/L
A540 ✓	<u>1-11-83 MF</u>	<u>0.020</u>
HPIC ✓		
37°C Light Scatter ✓		
IEP ✓	<u>JP</u>	

TESTING CHECK LIST

Lot # FFA1-3-45-4x1

Fractionation Sample	Protein			Cyto		Hem	
	Total	EGG	% Total	10 mc/ml	As Is	10 mc/ml	As Is

BOTTLING

	Date/Initial	
<u>Bottling Assembly</u>	<u>12-21-82</u>	<u>LN</u>
<u>Autoclaving</u>	<u>12-21-82</u>	<u>CL</u>
<u>Sterile Bottling</u>	<u>12-23-82</u>	<u>JP-W</u>
<u>Labelling</u>		
<u>Bact-T Samples Sent</u>		
<u>Pre-filter lot #</u>	<u>SLK 7002NRP N4840247</u>	
<u>Final filter lot #</u>	<u>DFA NRP, 2u</u>	
	<u>N 689021a</u>	
	<u>N 6890200</u>	

<u># Vials Bottled</u>	
<u>Total:</u>	<u>20 x 20cc 5 x 500cc</u>
<u>Sterility:</u>	<u>1 20cc</u>
<u>Potterity:</u>	
<u>Testing:</u>	
<u>To Ship:</u>	<u>19 x 20cc</u>
<u>Counted By:</u>	<u>PV & LN</u>
<u># Grams Bottled</u>	
<u>Total:</u>	
<u>To Ship:</u>	

Final Product Record Sheet

Lot # FFAI-3-45-4X1

Re Bottled 1-7-'83

	date, initials	Results
PHSS volume	<u>1/7/83 NW</u>	<u>2.51 ml</u>
Conductivity	<u>1/7/83 NW</u>	<u>11.4 mS @ 21°C</u>
pH	<u>1/7/83</u>	<u>6.76</u>
Protein	<u>1-7-83 MF</u>	<u>81.1 mg/ml g/vial</u>
Date Bottled	<u>1-7-83 JP W</u>	<u>125 ~ 350 ~ 200 ~</u> Vials - Total: <u>42</u> To Ship:
		Grams - Total: To Ship:

Assay	date, initials	
Cytotoxicity	<u>not done</u>	10 mg/ml: As Is:
Hemagglutination	<u>not done</u>	10 mg/ml: As Is:
Pyrogen (610.13b)✓	<u>1-17-83 JP</u>	pass: <u>X</u> fail: Av. High Dev.: .1
Platelet Aggregation ✓	<u>1-17-83 JP</u>	# seconds at 10 mg/ml <u>NO Δ</u>
Safety (610.11)✓	<u>1-7-1983 PV</u>	pass: <u>X</u> fail:
HAA (610.40)	<u>not done</u>	Antigen: (+) or (-)
IEP ✓	<u>1-12-1983</u>	Antibody: (+) or (-)

Sterility (610.12)✓	<u>ECO 1-7-83</u>	Sterile: <u>X</u> Nonsterile:
ECG ✓	<u>1-24-83 JP</u>	<u>2.9 mg/ml</u>
Mixed Agglutination	<u>not done</u>	1: at 10 m
AS40 ✓	<u>1-11-83 MF</u>	<u>0.012</u>
HPLC ✓	<u>1-7-83 JP</u>	
37°C Light Scatter ✓		
IEP ✓	<u>1-11-83 JP</u>	<u># 333</u>

TESTING CHECK LIST

Lot # FFAI-3-45-4X1
Rebottled 1-7-83

Fractionation Sample	Protein			Cyto		Hem	
	Total	EGG	% Total	10 mc/ml	As Is	10 mc/ml	As Is
<u>Final Product</u>		<u>2.9</u>					
<u>Plasma Tot</u>		<u>9.2</u>					

BOTTLING

Date/Initial

Bottling Assembly 1-6-83 LN

Autoclaving 1-6-83 CR

Sterile Bottling 1-7-83 LN, JP

Labelling 1-7-83

Bact-T Samples Sent ECO 1-7-83 6N

Pre-filter lot # Seal Clean N4840160

Final Filter lot # DFA NRP B097241 2m
approx. 20cc/vial, ≤ 5000 cc/bottle
bottled w/ bell + hemostat.

~3x500cc
~2x200cc

Vials Bottled

Total: 42x20cc

Sterility: 2x20cc 1st & Last

Posteriority:

Testing: 2x20cc

To Ship:

Counted By:

Grams Bottled

Totals:

To Ship:

Botulism Antitoxin, Heptavalent
(EQUINE). F(ab'),
20cc per vial, STERILE
75.3 mg/ml Bottled 4/21/83
Lot #FFA2-3-45-4X1

Final Product Record Sheet

Lot # FFA2-3-45-4X1

Bottled 4-21-83

	date, initials	Results
PHSS volume	4-21-83 <i>RJ</i>	7.75 L
Conductivity	4-21-83 <i>RJ</i>	11.7 mS @ 23°C
pH	4-21-83 <i>RJ</i>	6.76
Protein	4-21-83 <i>MF</i>	75.3 mg/ml 1.51 g/vial F-51
Date Bottled	4-21-83 <i>LN + JP</i>	Vials - Total: To Ship:
		Grams - Total: 5836 To Ship:

↳ Based on pre bottle *rel. assay*

Assay	date, initials	
Cytotoxicity	_____	10 mg/ml: As Is:
Hemagglutination	_____	10 mg/ml: As Is:
Pyrogen (610.13b)	4/28/83 <i>SN</i>	pass: <input checked="" type="checkbox"/> fail: Av. Hqd. Dev.: 0.180
Platelet Aggregation	1-5-21-83 <i>JP</i>	# seconds at 10 mg/ml <i>75.3 mg/ml No d</i>
Safety (610.11)	4-27-83 <i>PV</i>	pass: <input checked="" type="checkbox"/> fail:
HA (610.40)	_____	Antigen: (+) or (-)
DP	4-26-83 <i>LN</i>	Antibody: (+) or (-)

mg/ml

		Sterile:	Nonsterile:
Sterility (610.12)	<i>ECO</i>		
NO2	5-2-83 <i>JP</i>	6.4 mg/ml <i>infect</i>	<i>Flu</i>
Mixed Agglutination	_____	1: _____ at 10 mg/ml	
A540	4-26-83 <i>MF</i>	Pre C: 0.22	Final Product: 0.21
MPIC	5-5-83 <i>TI</i>	see graph	
37°C Light Scatter	5-6-83 <i>UL</i>	see graph	
DP	5-5-83 <i>JP</i>	#341	

TESTING CHECK LIST

Lot # FFA2-345-4x1

<u>Fractionation Sample</u>	<u>Protein</u>			<u>Cyto</u>		<u>Hem</u>	
	<u>Total</u>	<u>ECG</u>	<u>% Total</u>	<u>10 mg/ml</u>	<u>As Is</u>	<u>10 mg/ml</u>	<u>As Is</u>
<u>Plasma Pool</u>	<u>51.3</u>	<u>11.1</u>	<u>21.6%</u>				
<u>Post-Strain Filter Pool</u>		<u>9.6</u>					
<u>Pre-QAG A</u>	<u>59.6</u>	<u>3.3</u>	<u>5.5%</u>				
<u>Pre-QAG C</u>	<u>50.4</u>	<u>4.2</u>	<u>8.3%</u>				
<u>F.P.</u>	<u>75.3</u>	<u>6.4</u>	<u>8.5%</u>				

BOTTLING

<u></u>	<u>Date/Initial</u>	<u># Vials Bottled</u>
<u>Bottling Assembly</u>	<u>4-18-83 LN</u>	
<u>Autoclaving</u>	<u>4-19-83 CR</u>	<u>Total: 9 vials 17 500cc</u>
<u>Sterile Bottling</u>	<u>4-21-83 LN/SP</u>	<u>Bact-T: 2 15 w/ 500cc</u>
<u>Labelling</u>	<u>4-26-83 PV</u>	<u>Posterity: 0 1 w/ 350cc</u>
<u>Bact-T Samples Sent</u>	<u>ECO 5-3-83</u>	<u>Testing: 2 1 w/ 125cc</u>
<u>Pre-filter lot # 16018P R1030MT</u>		<u>To Ship: 5</u>
<u>Final filter lot # 1474301AMP R473062</u>		<u>Counted By:</u>
<u>R473062</u>		<u># Grams Bottled</u>
		<u>Total:</u>
		<u>To Ship:</u>



TECHNICAL DATA

STERILITY AUDIT REPORT

STERILITY AUDIT REPORT TO: UNIVERSITY OF MINNESOTA

DATE RECEIVED: 12/30/1982 - 1st Time bottled. Rebottled 1-7-8

DATE TEST ENDED: 1/13/1983

SAMPLE DESCRIPTION: BIOLOGICS

LOG NUMBER: 14500

REFERENCE: [21 CFR PART 610.12]

ITEM	LOT NO.	NUMBER TESTED	MEDIA TG SCD	INCUBATION DAYS TEMP.	POSITIVE TG SCD
BOTULISM ANTITOX.FFA	13454X1	2	1 1	14 32/22	0 0

SCD - SOYBEAN CASEIN DIGEST
TG - FLUID THIOGLYCOLLATE

THESE SAMPLES PASS THE TEST FOR STERILITY

It is the client's responsibility to have on file test data indicating the bacteriostatic/fungistatic characteristics of the product

COMMENTS :

Capsule Laboratories

Lynn Long

Lynn Long
Microbiologist

CAPSULE LABORATORIES

Appendix IVa



TECHNICAL DATA

STERILITY AUDIT REPORT

STERILITY AUDIT REPORT TO: UNIVERSITY OF MINNESOTA

DATE RECEIVED: 1/10/1983

DATE TEST ENDED: 1/26/1983

SAMPLE DESCRIPTION: BIOLOGICS

LOG NUMBER: 14535

REFERENCE: [21 CFR PART 610.12]

ITEM	LOT NO.	NUMBER TESTED	MEDIA		INCUBATION		POSITIVE	
			TG	SCD	DAYS	TEMP.	TG	SCD
FFA1-3-45-4X1	1/7/83	4	2	2	14	32/22	0	0
FFA1 RERUN PRE QAE	1/6/83	2	1	1	14	32/22	0	0
FFA1 RERUN POST QAE	1/6/83	2	1	1	14	32/22	0	0

SCD - SOYBEAN CASEIN DIGEST
TG - FLUID THIOGLYCOLLATE

THESE SAMPLES PASS THE TEST FOR STERILITY

It is the client's responsibility to have on file test data indicating the bacteriostatic/fungistatic characteristics of the product

COMMENTS :

Capsule Laboratories

Lynn Long
Microbiologist

CAPSULE LABORATORIES

Appendix IVb



TECHNICAL DATA

STERILITY AUDIT REPORT

STERILITY AUDIT REPORT TO: UNIVERSITY OF MINNESOTA

DATE RECEIVED: 4/22/1983

DATE TEST ENDED: 5/ 6/1983

SAMPLE DESCRIPTION: BIOLOGICS

LOG NUMBER: 14961

REFERENCE: [21 CFR PART 610.12]

ITEM	LOT NO.	NUMBER TESTED	MEDIA		INCUBATION		POSITIVE	
			TG	SCD	DAYS	TEMP.	TG	SCD
PFA2-3-45-4X1	4/21/83	4	2	2	14	32/22	0	0

SCD - SOYBEAN CASEIN DIGEST
TG - FLUID THIOGLYCOLLATE

THESE SAMPLES PASS THE TEST FOR STERILITY

It is the client's responsibility to have on file test data indicating the bacteriostatic/fungistatic characteristics of the product

COMMENTS :

Capsule Laboratories

Lynn Long
Microbiologist

CAPSULE LABORATORIES

Appendix V

PYROGEN TEST

Sample Tested: Botulism Antitoxin, Heptavalent
 Protein (mg/ml): 77.8 (Eq) Flab)

Date: 12-29-82 By: JP

FFA1-3-45-4x1 (Lot #)

Rabbit Baseline Temperatures

MIN	TIME	# 10	# 11	# 12	# 13	#
0'	9:15	38.7	38.95	38.75	38.65	
15'	9:30	38.65	38.85	38.75	38.6	
30'	9:45	38.55	39.0	38.6	38.5	
45'	10:00	38.5	39.15	38.5	38.45	
60'	10:15	38.5	39.2	38.55	38.45	
75'						
90'						

Rabbit Number	Weight - Pounds	cc Sample Injected
10	4-1	1.83
11	4-1	1.83
12	5-6	2.42
13	4-1	1.83

Undiluted (1mg/kg)
 Diluted to 5.0cc

with non-pyrogenic saline

1ml/kg
 $.45\text{kg/lb} \times \text{wt. (lbs)} \times \text{dose}$
 mg/ml (protein conc. of sample)

Rabbit Temperatures - Post Injection

MIN	TIME	# 10	# 11	# 12	# 13	#
0	10:20	38.5	39.2	38.7	38.4	
30	10:50	38.55	39.1	38.75	38.4	
60	11:20	38.5	39.1	38.85	38.4	
90	12:20	38.55	39.15	38.9	38.4	
120	1:20	38.65	39.0	38.95	38.45	

Sample Tested: FFA1-3-45-4X1

Date Bottled: 12-23-82

PYROGEN TEST RECORD SHEET

Times Tested: 1) X 2) 3)

Date

Conditioned: 12-28-82 By: JP

Date Tested: 12-29-82 By: JP

Rabbit #	CC Injected	Pre	30'	60'	120'	180'	Avg. Rise	High Deviation
10	1.83	38.5	38.55	38.5	38.55	38.65	.063	.15
11	1.83	39.2	39.1	39.1	39.15	39.0	0	0
12	2.42	38.55	38.75	38.85	38.9	38.95	.313	.4
13	1.83	38.45	38.4	38.4	38.4	38.45	0	0

Group Avg. Rise: .091

Group Avg. of Highest Deviation: .138

Passed: X Failed:

Comments: No adverse rx's seen in any of the test rabbits

PYROGEN TEST

Sample Tested: FFA1-3-45-4x1

Date: 1-17-88 By: JP

Protein (mg/ml): 81.9

Rabbit Baseline Temperatures

MIN	TIME	# 10	# 11	# 12	# 13	#
0'	8:05	38.85	39.0	39.35	39.0	
15'	8:20	38.8	38.95	39.25	38.9	
30'	8:35	38.7	38.85	39.2	38.9	
45'	8:50	38.7	38.95	39.15	38.9	
60'	9:05	38.75	38.9	39.15	38.9	
75'						
90'						

Rabbit Number	Weight-Pounds	cc Sample Injected
10	4-5	1.94
11	3-8	1.58
12	3-14	1.75
13	3-11	1.66

14, undiluted
Diluted to 5.0cc

with non-pyrogenic saline
1ml/kg
[.45kg/lb x wt. (lbs) x 1ml/kg dose
mg/ml (protein conc. of sample)]

Rabbit Temperatures - Post Injection

MIN	TIME	# 10	# 11	# 12	# 13	#
0	9:10	38.8	39.05	39.2	39.0	
30	9:40	39.0	39.05	39.4	39.15	
60	10:10	39.1	39.15	39.5	39.25	
120	11:10	39.1	39.15	39.35	39.25	
180	12:10	39.0	39.1	39.3	39.2	

Sample Tested: FFA1-3-45-4x1

Date Bottled: 1-9-83

PYROGEN TEST RECORD SHEET

Times Tested: 1) X 2) 3)

Date Conditioned: 1-17-83 By: JP

Date Tested: 1-17-83 By: JP

Rabbit #	cc Injected	Pre	30'	60'	120'	180'	Avg. Rise	High Deviation
10	1.94	38.75	39.0	39.1	39.1	39.0	.3	.35
11	1.58	38.9	39.05	39.15	39.15	39.1	.213	.25
12	1.75	39.15	39.4	39.5	39.35	39.3	.238	.35
13	1.66	38.9	39.15	39.25	39.25	39.2	.3	.35

Group Avg. Rise: .263

Group Avg. of Highest Deviation: .325

Passed: X Failed:

Comments: 2nd pyrogen test on FFA1-3-45-4x1, reprocessed for 2nd bottling, ~20 vials for possible human use.

PYROGEN TEST

(EQUINE), Hcb¹/₂

Sample Tested: Botulinus Antib toxin, Hepta valent

Date: 4/28/83 By: SN

Protein (mg/ml): 75.3 mg/ml Lot # FFA2-3-45-4X1

Rabbit Baseline Temperatures

MIN	TIME	#10	#11	#12	#13	#14
0'	1:20	37.7	39.4	39.9	39.8	39.85
15'	1:35	39.4	39.2	39.7	39.7	39.7
30'	1:50	39.35	39.05	39.55	39.5	39.55
45'	2:05	39.25	39.00	39.35	39.45	39.3
60'	2:20	39.20	38.85	39.25	39.45	39.15
75'	2:35	39.20	38.80	39.20	39.40	39.05
90'						

Rabbit Number	Weight-Pounds	cc Sample Injected
10	7 lbs 10 oz	3.43 ml
11	5 lbs 5 1/2 oz	2.40
12	8 lbs 3 1/2 oz	3.70
13	8 lb 20 oz	3.66
14	6 lbs	2.70

Diluted to 5.0cc
with non-pyrogenic saline
1 ml
(.45kg/1h x wt. (1lb) = 20mg/kg-dose
mg/ml (protein conc. of sample)

Rabbit Temperatures - Post Injection

MIN	TIME	#10	#11	#12	#13	#14
0	3:10	39.65	39.2	39.65	39.6	38.9
30	3:40	39.3	39.0	39.50	39.6	38.9
60	4:10	39.95	38.9	39.35	39.40	38.9
120	5:10	39.20	39.1	39.35	39.35	38.9
180	6:10	39.15	38.95	39.35	39.40	39.0

Station Ant. Unit, Dept
Sample Tested: Lot # FFA2-3-45-4X1

Date Bottled: 4/21/83

PYROGEN TEST RECORD SHEET

Times Tested: 1) ☒ 2) ☐ 3) ☐

Date Conditioned: 4/27/83 By: EN

Date Tested: 4/28/83 By: EN

Rabbit #	CC Injected	Pre	30'	60'	120'	180'	Avg. Rise	High Deviation
10	3.43 mL	39.20	39.30	39.25	39.20	39.15	.017 0.009	0.05 0.1
11	2.40	38.80	39.0	38.90	39.10	38.95	.113 0.170	0.3
12	3.70	39.20	39.50	39.35	39.35	39.35	0.150	0.15 0.2
13	3.66	39.40	39.60	39.40	39.35	39.40	0.030	0.2
14	2.70	39.05	38.90	38.90	38.90	39.0	0	0.00

Group Avg. Rise: 0.070 ~~0.070~~ 0.10

Group Avg. of Highest Deviation: 0.180 ~~0.180~~ 0.1

Passed: ☒ Failed: ☐

Comments: _____

SAFETY TEST

LOT # FFAI-3-45-4X1
BOTTLED 1-7-83

GUINEA PIGS 5.0cc I.P. each

Received 1-5-83Test started 1-7-83 By PV Test ended 1-14-83 By PV

Description	Pre injection weight	Weight end of test	
#1 <u>white</u>	<u>250g</u>	<u>300g</u>	<u>Pass</u> or Fail
#2 <u>Br/white</u>	<u>265g</u>	<u>315g</u>	<u>Pass</u> or Fail

MICE .5cc I.P. each

Type SW ♀ Rcd 1-6-83Test started 1-7-83 By PV Test ended 1-14-83 By PV

	Pre injection weight	Weight at end of test	
#1 Not clipped	<u>18.0g</u>	<u>22.9g</u>	<u>Pass</u> or Fail
#2 Clipped	<u>17.8g</u>	<u>22.0g</u>	<u>Pass</u> or Fail

SAFETY TEST

LOT # FFA2-3-45-4X1

GUINEA PIGS 5.0cc I.P. each

Received 4-26-83Test started 4-27-83 By PV Test ended 5-4-83 By PV

Description	Pre injection weight	Weight end of test	
#1 <u>Black</u>	<u>290g</u>	<u>330g</u>	<u>Pass</u> or Fa
#2 <u>Bl/white</u>	<u>270g</u>	<u>325g</u>	<u>Pass</u> or Fa

MICE .5cc I.P. each

Type SW ♀Test started 4-27-83 By PV Test ended 5-4-83 By PV

Pre injection weight	Weight at end of test	
#1 Not clipped	<u>21.4g</u>	<u>24.1g</u> <u>Pass</u> or Fai
#2 Clipped	<u>20.6g</u>	<u>24.2g</u> <u>Pass</u> or Fai

Despeciation Apparatus for Small Scale Experiments

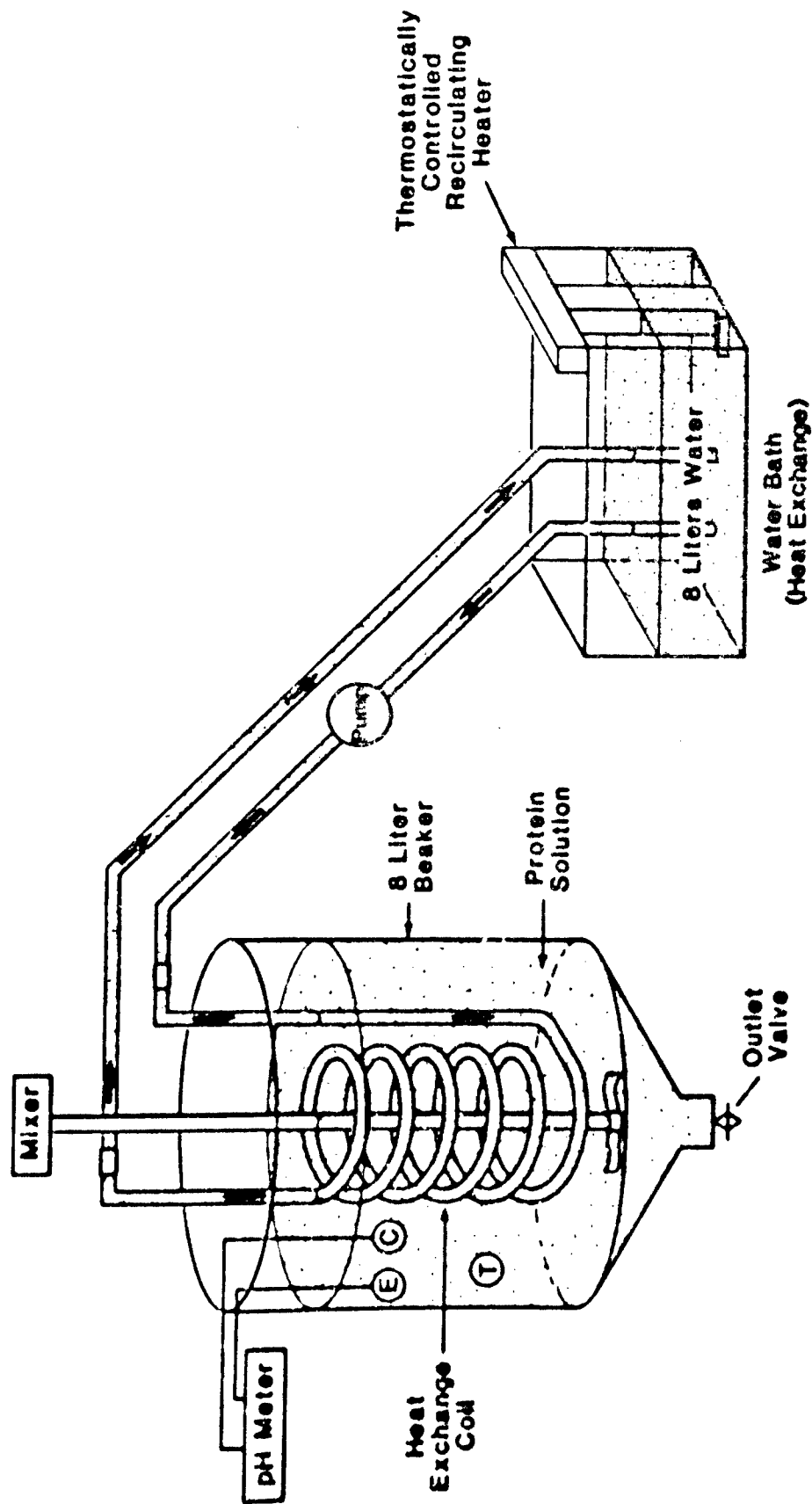


Figure 1
69

- Ⓓ Thermometer
- Ⓔ Combination pH Electrode
- Ⓒ Automatic Temperature Compensator

Despeciation Equipment for FFA1-3-45-4x1 and FFA2-3-45-4x1

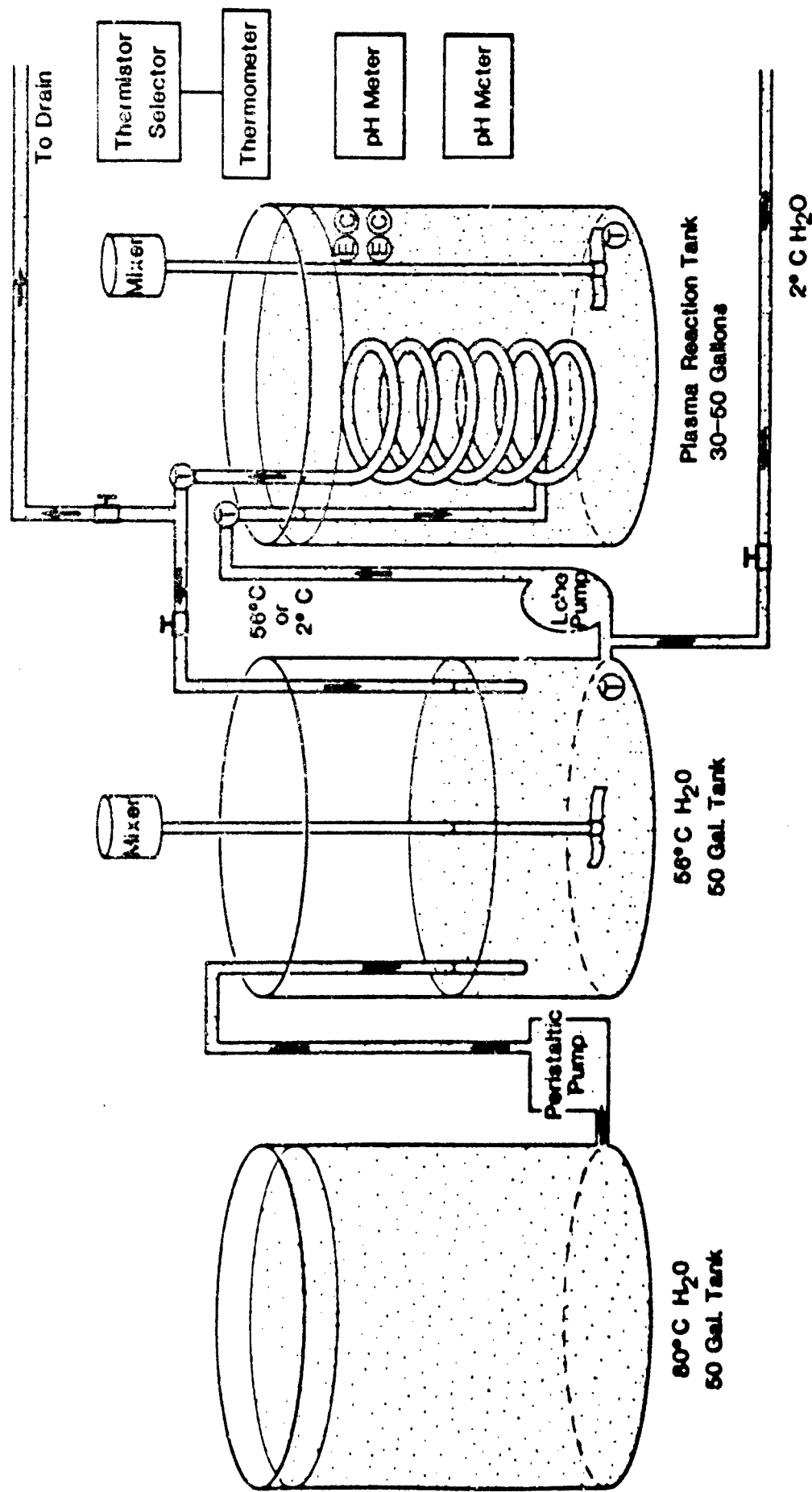
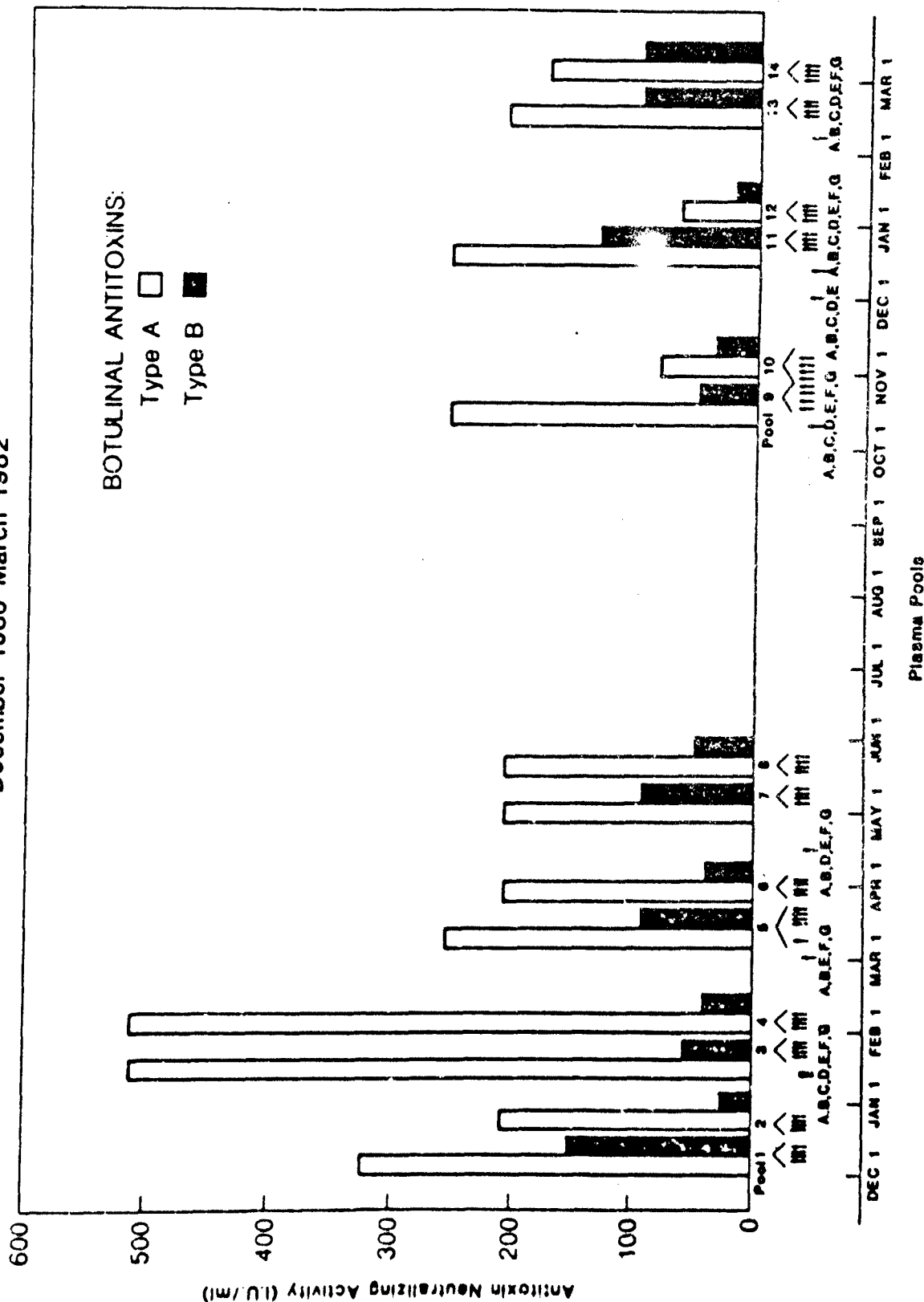


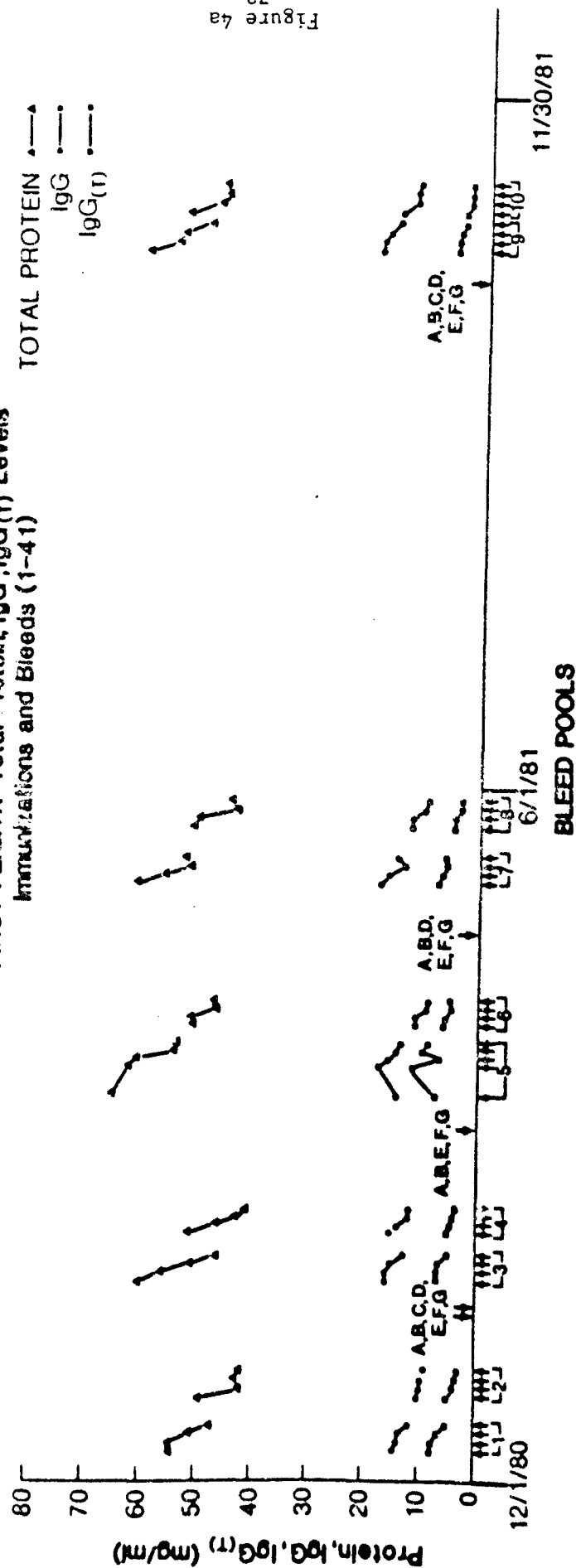
Figure 2
70

C. BOTULINAL ANTITOXIN NEUTRALIZING ACTIVITY (TYPE A AND TYPE B)
 First Flight Plasma Pools (1-14)
 December 1980-March 1982



FIRST FLIGHT: Total Protein, IgG, IgG(r) Levels
Immunizations and Bleeds (1-41)

TOTAL PROTEIN —▲—
IgG —●—
IgG(r) —○—



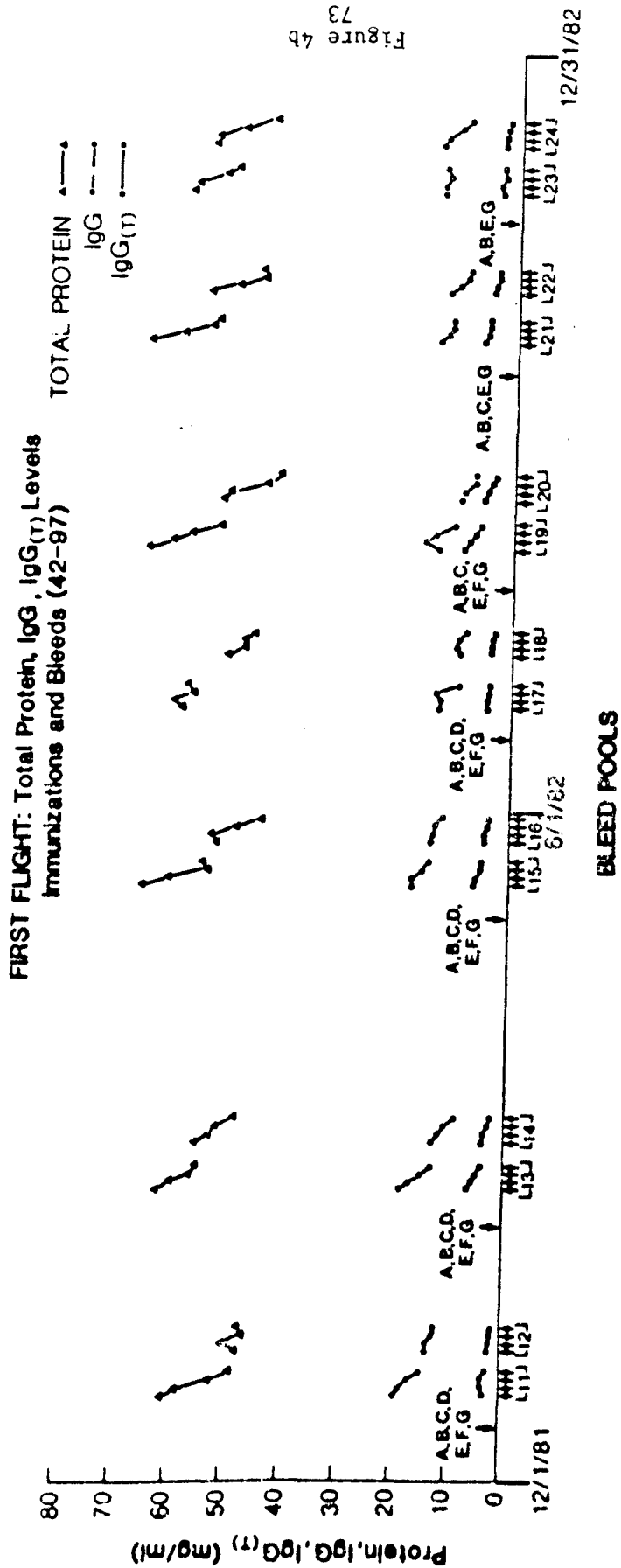
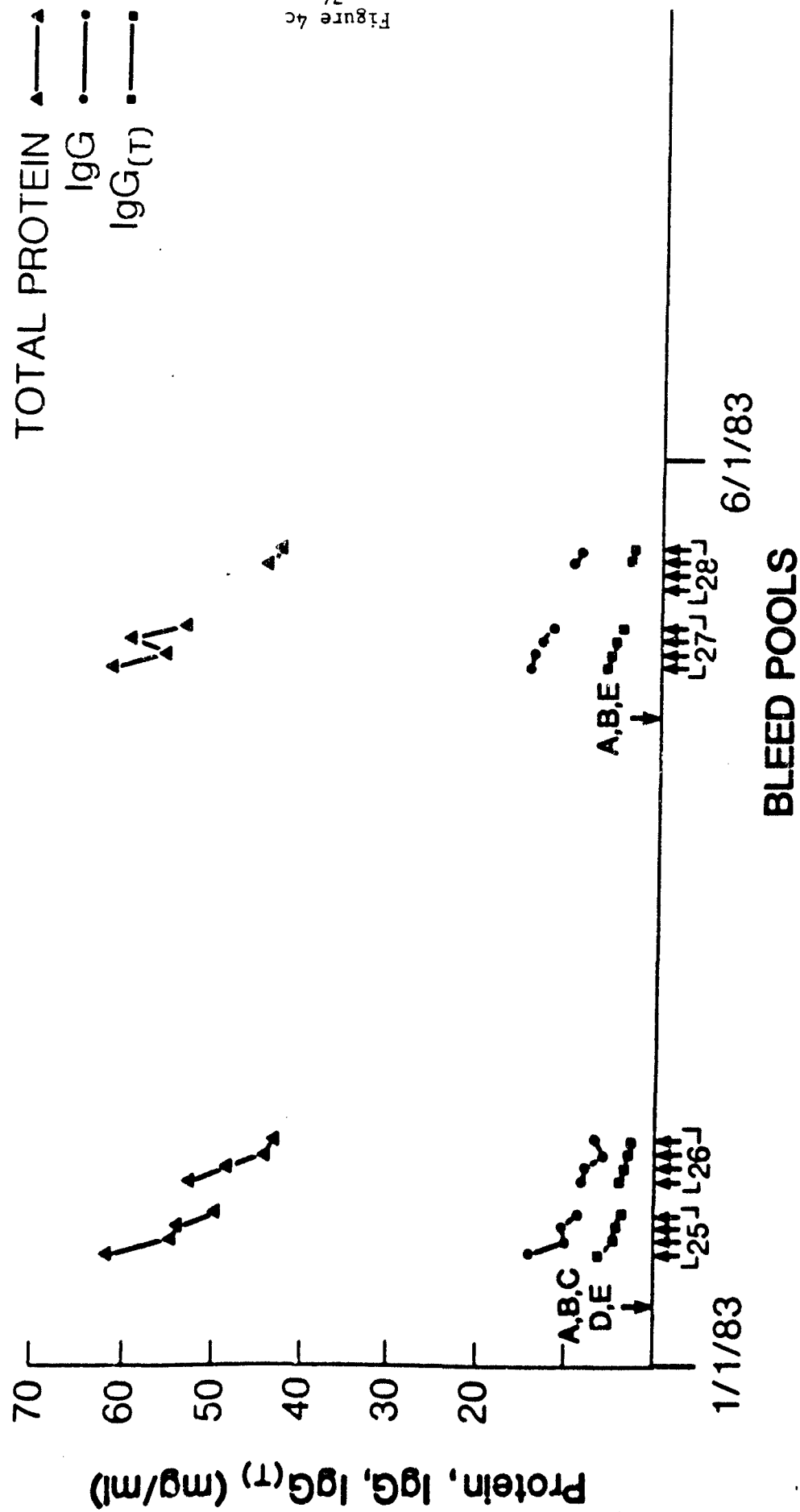
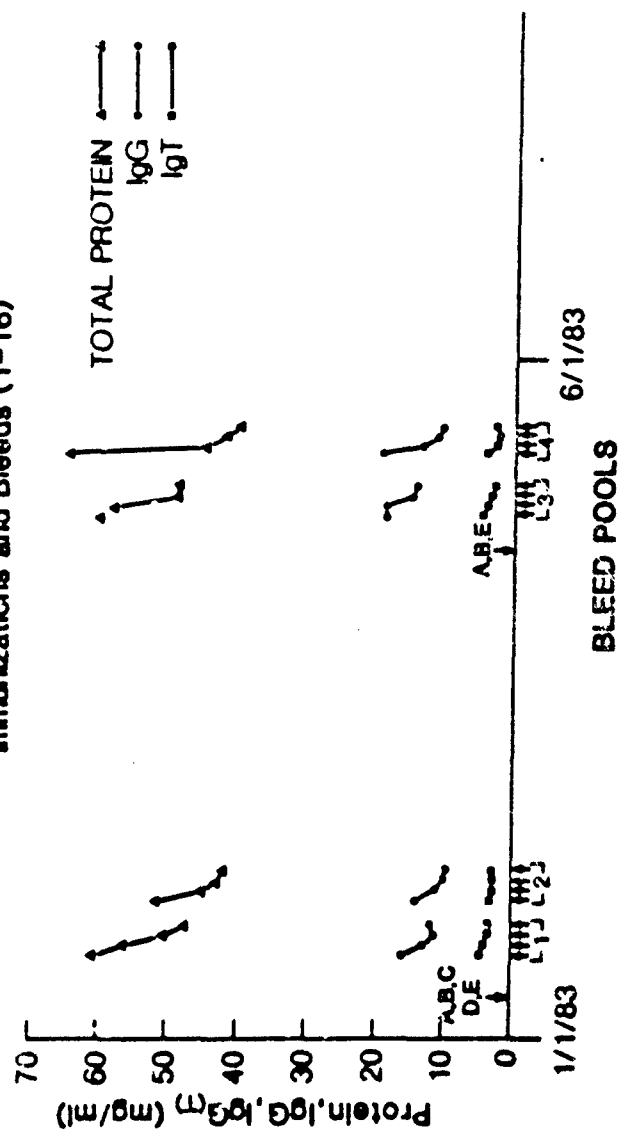


Figure 4b
73

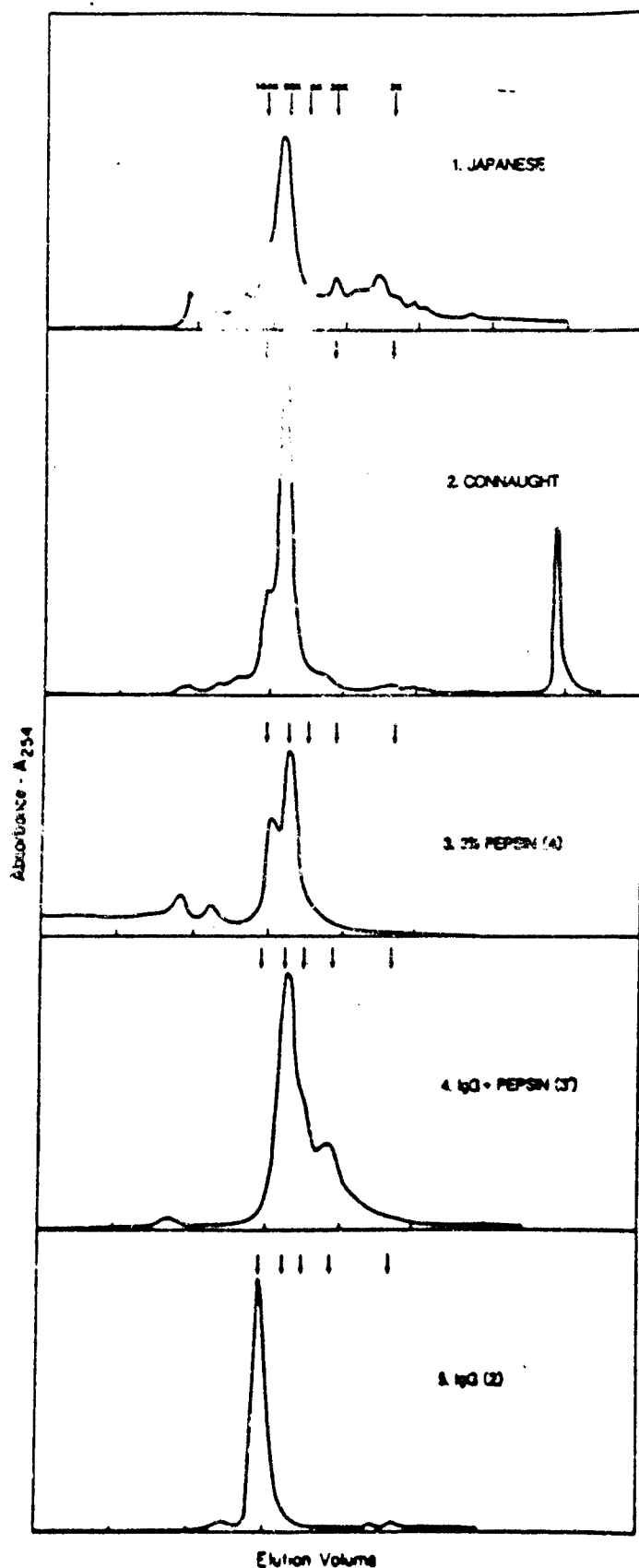
FIRST FLIGHT: Total Protein, IgG, IgG_(T) Levels Immunizations and Bleeds (98-113)



ABE: Total Protein, IgG, IgG_(T) Levels
Immunizations and Bleeds (1-16)



Molecular Composition of Equine Botulinal Antitoxins:



Elution Volume

Figure 6

FFA1-3-45-4x1
Temperature and pH During Despeciation

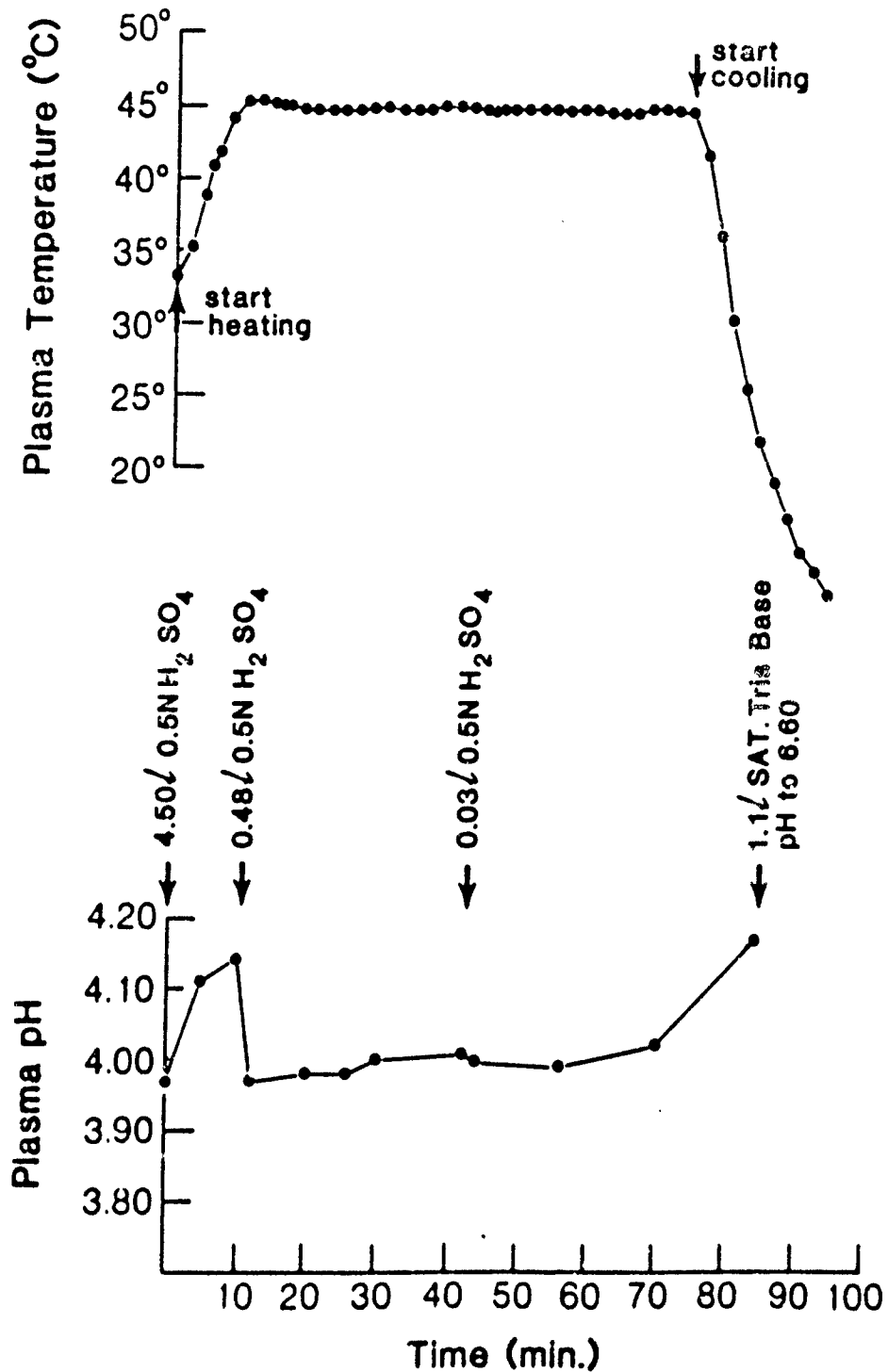


Figure 7
77

FFA2-3-45-4x1

Temperature and pH During Despeciation

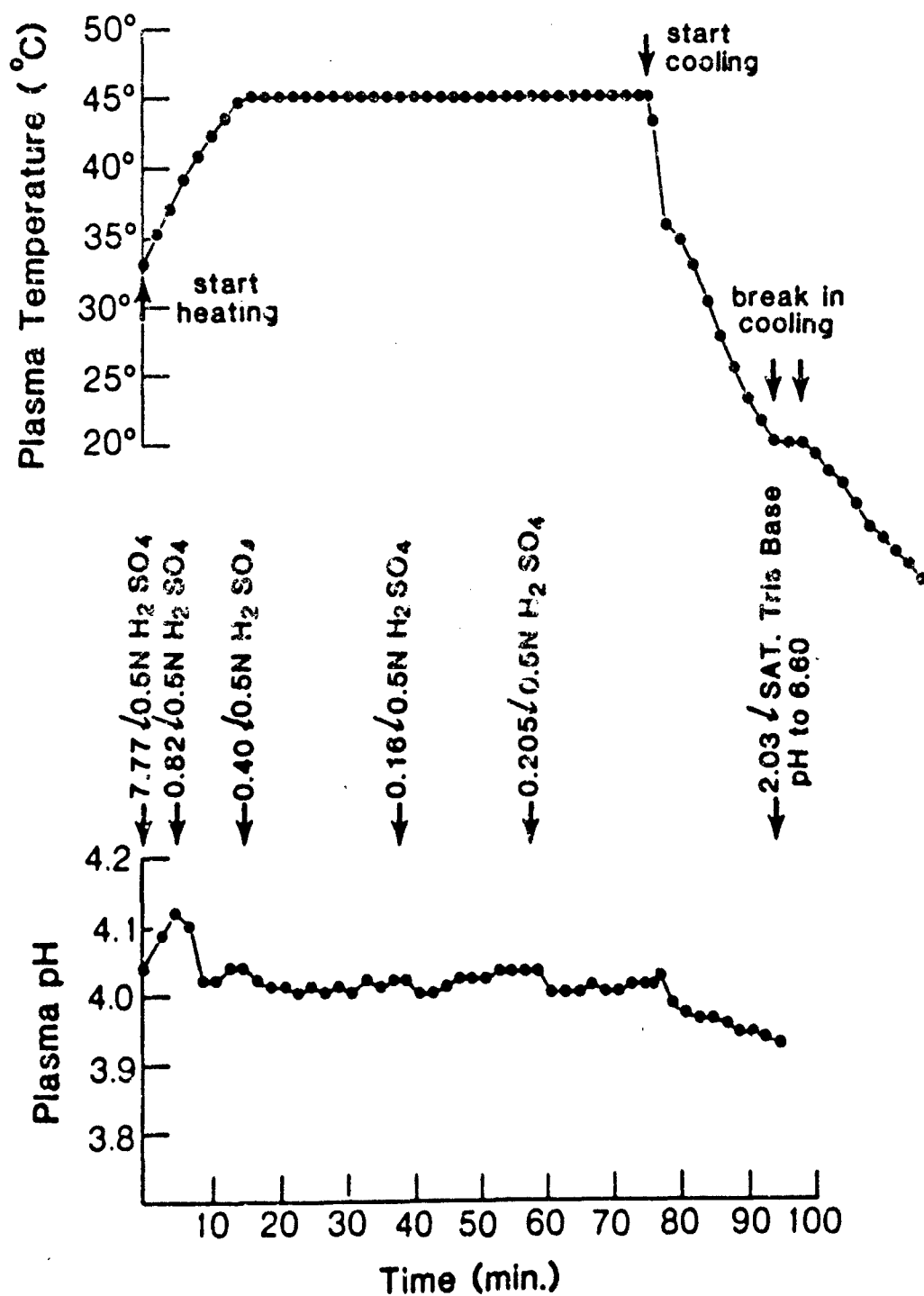


Figure 8
78

**HPLEC Analysis of the Critical Steps
During the Preparation of Equine F(ab')₂
FF A1-3-45-4x1**

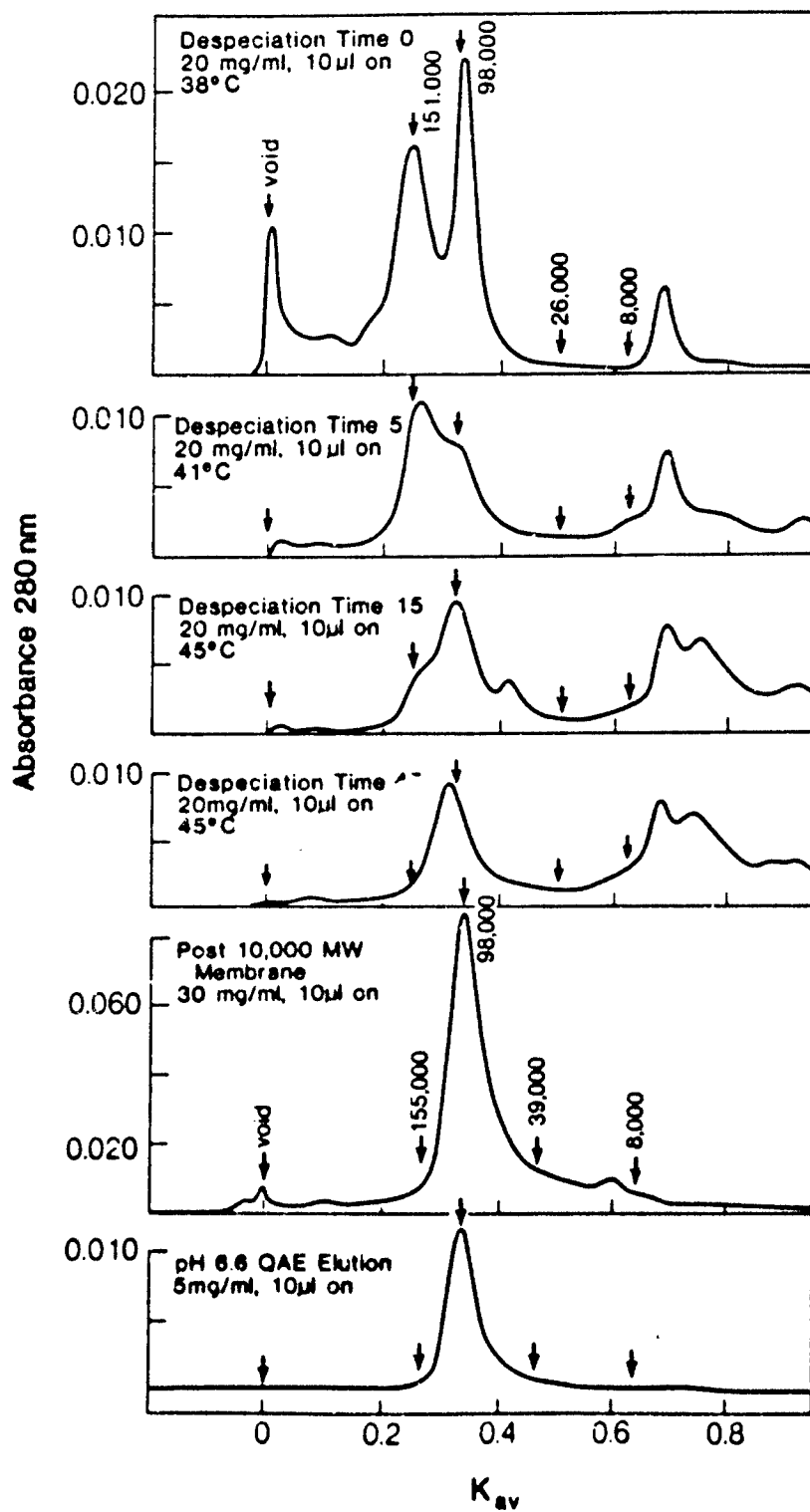


Figure 9
79

HPLEC Analysis of the Critical Steps
During the Preparation of Equine F(ab')₂
FF A2-3-45-4x1

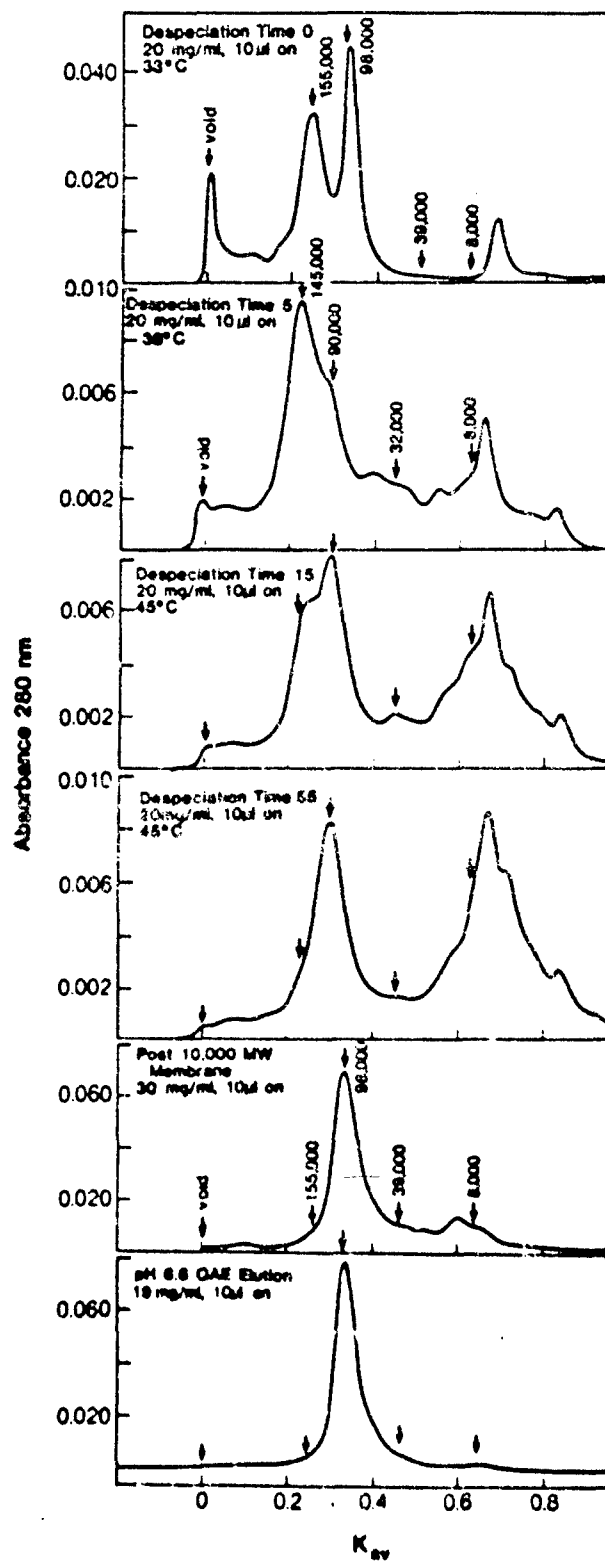


Figure 10
80

4 x 1 1/2

Botulism Antitoxin, (Fab')₂, Heptavalent

EQUINE For Parenteral Use

Each 10ml vial contains:	Type A > 16,340 IU/ml
	Type B > 7,660 IU/ml
	Type C > 2,370 IU/ml
	Type D > 115,710 IU/ml
	Type E > 12,800 IU/ml
	Type F > 1,410 IU/ml
	Type G > 5,120 IU/ml

Contains: Limited by Federal Law to Investigational Use
 Preserved by Addition of Benzalkonium Chloride, at 0.01% w/v. Aqueous Solution. Contains 0.5% Benzalkonium Chloride.

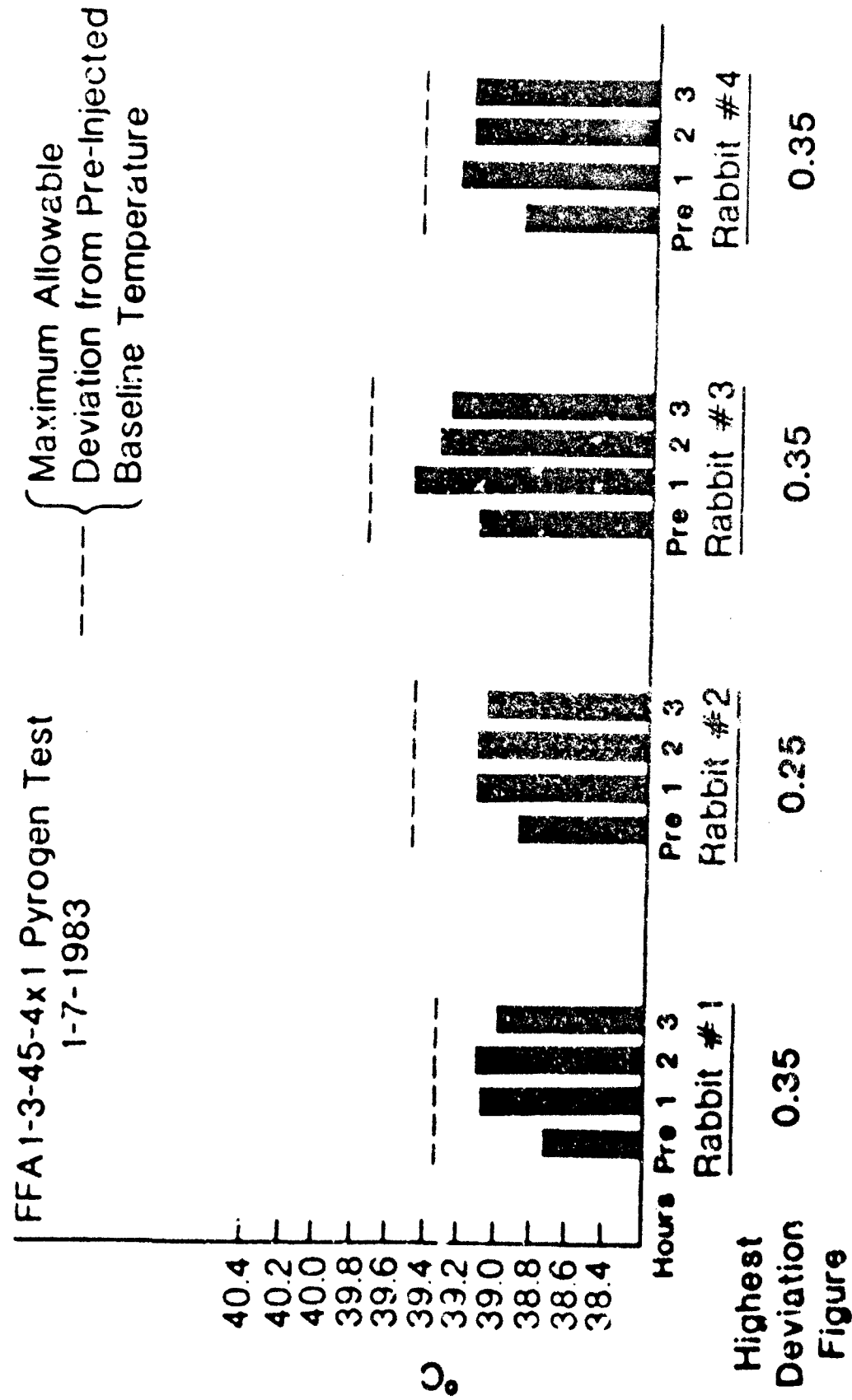
Keep at 2-8°C
 Do not freeze
 Do not shake

Lot # M42-3-45-47

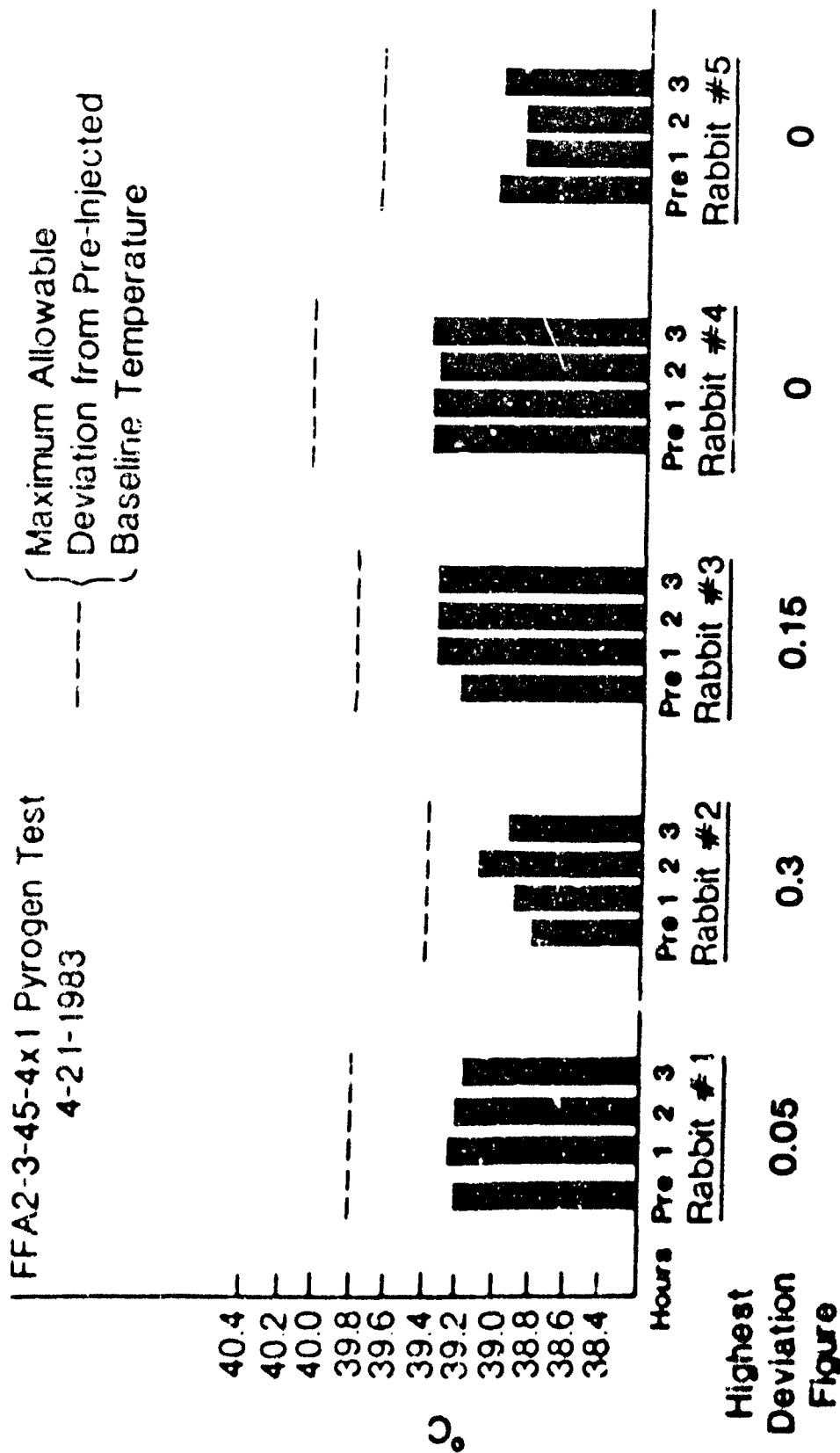
Expiration Date

Job No. 44382,

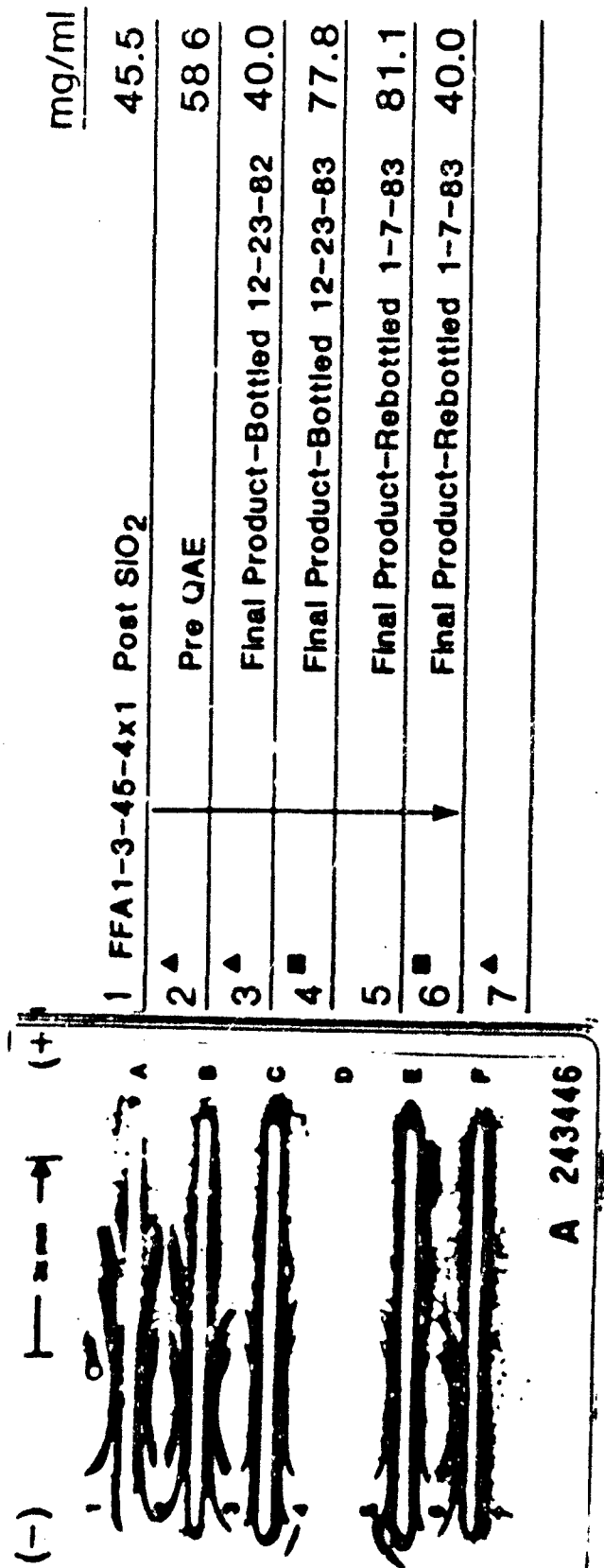
Pyrogen Testing of FFA 1-3-45-4x1 Final Product



Pyrogen Testing of FFA2-3-45-4x1 Final Product



Immunoelectrophoresis of FFA 1-3-45-4x1 Final Product

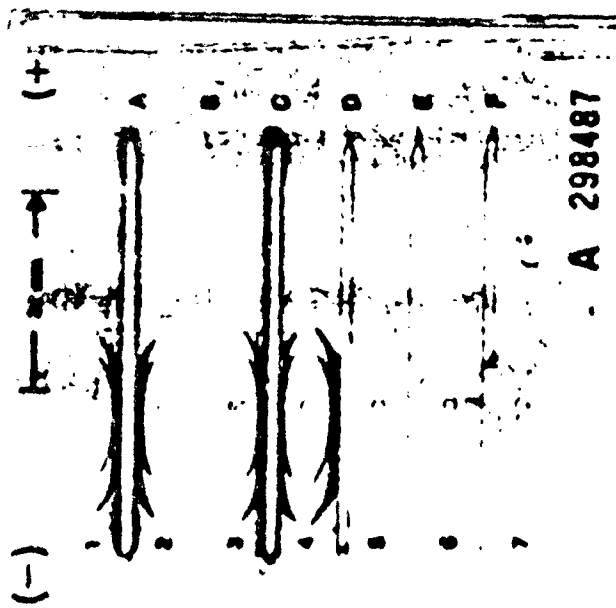


Samples Electrophoresed for 80 Minutes at 80 Volts

Antisera: ▲ Miles, Rabbit Anti -Equine IgG, Lot #R491

■ Kallestad, Rabbit Anti-Whole Equine(1:2 w/NaCl), Lot #R678

Immunoelectrophoresis of FFA2-3-45-4x1 Final Product



	FFA2-3-45-4x1	Pre QAE C	mg/ml
1			40.0
2			40.0
3			40.0
4			40.0
5			75.3
6			
7			

Figure 15
85

Samples Electrophoresed for 80 Minutes at 80 Volts
 Antisera: ▲ Gibco, Rabbit Anti-Whole Equine (1:2 w/NaCl), Lot #28P8C23
 ■ Miles, Rabbit Anti-Equine IgG (1:2 w/NaCl), Lot #R678

Large Scale Despeciation of First Flight B(1-57) Plasma by Pepsin

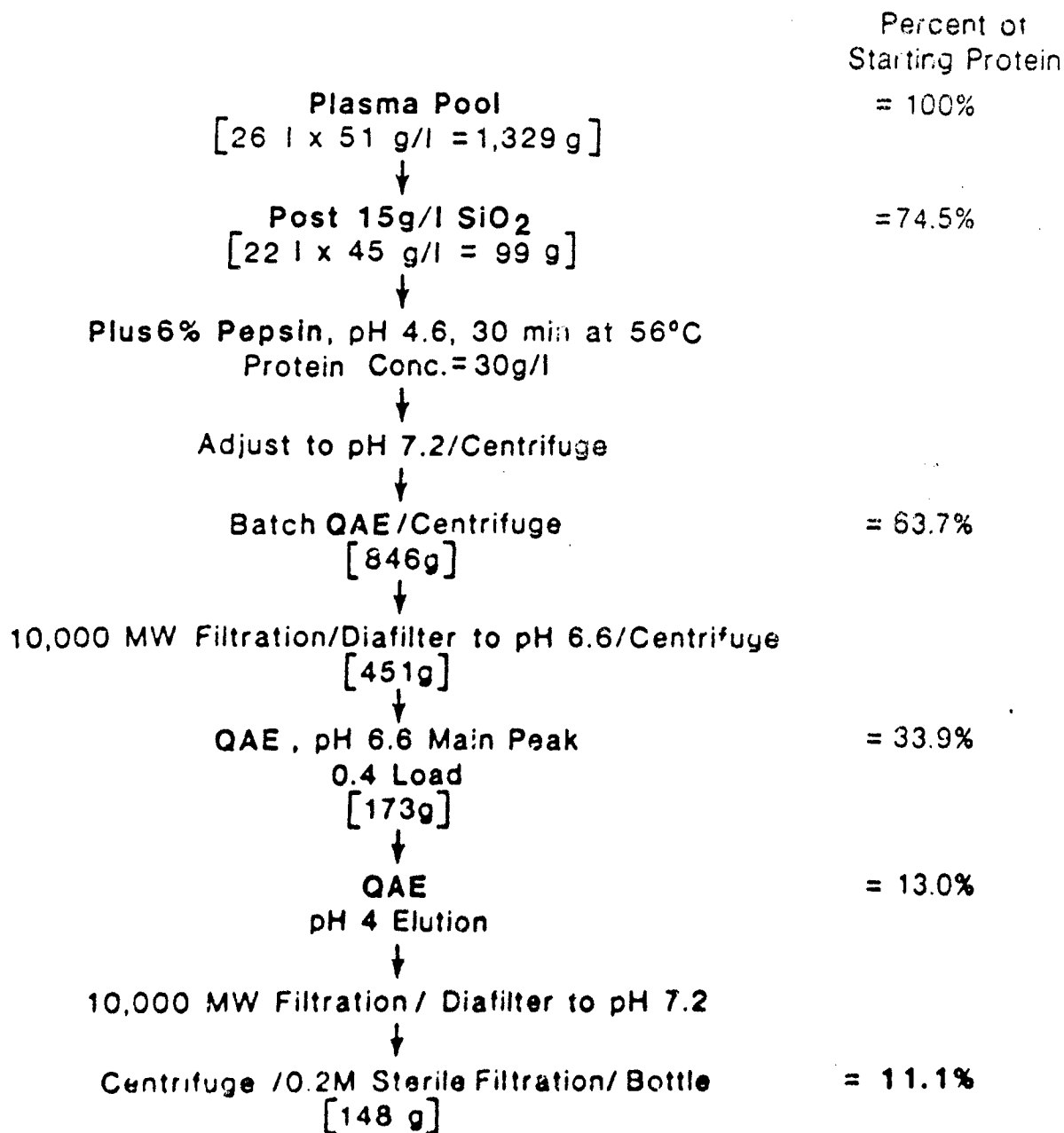


Table I

IMMUNIZATION SCHEDULE FOR "FIRST FLIGHT"

BETTERMENT DOTULINUM ANTITOXIN

Date	Day	A	B	C	D	E	F	G	CONTENT
26 Sep 78	1	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	50:150 Freund's MDPH Penta.
25 Oct 78	29	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	MDPH Penta.
15 Nov 78	50	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	MDPH Penta.
12 Dec 78	77	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	MDPH Penta.
4 Jan 79	100	-	Toxoid	-	-	-	-	-	MDPH Mono.
24 Jan 79	124	-	Toxoid	-	-	-	-	-	MDPH Mono.
24 Mar 79	183	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	MDPH Penta.
23 Jun 79	274	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	MDPH Penta.
25 Jul 79	304	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	MDPH Penta.
3 Aug 79	315	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	MDPH Penta.
18 Oct 79	381	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	MDPH Penta.
16 Nov 79	420	Toxoid	-	-	-	-	-	-	Neurotoxoid
18 Dec 79	453	-	-	-	-	-	-	Toxoid	50:150 Freund's
2 Jan 80	467	-	-	-	-	-	-	Toxoid	-
15 Jan 80	480	-	-	-	-	-	-	Toxoid	-
27 Jan 80	502	Toxoid	-	-	-	-	-	Toxoid	A-Neurotoxoid
13 Feb 80	509	-	-	-	-	-	-	Toxoid	-
21 Feb 80	517	Toxoid	-	-	-	-	-	Toxoid	-
27 Feb 80	523	-	-	-	-	-	-	Toxoid	-
4 Mar 80	529	-	-	-	-	-	-	Toxoid	-
11 Mar 80	536	-	-	-	-	-	-	Toxoid	-
18 Mar 80	543	-	-	-	-	-	-	Toxoid	-
26 Mar 80	551	-	-	-	-	-	-	Toxoid	-
3 Apr 80	554	-	-	-	-	-	-	Toxoid	-
14 Apr 80	576	-	Toxoid	-	-	Toxoid	-	Toxoid	B. & M. MDPH Mono.
9 May 80	585	-	-	-	-	Toxoid	-	Toxoid	-
14 May 80	604	-	-	-	-	Toxoid	-	Toxoid	-
17 Jun 80	636	-	-	Toxoid	-	Toxoid	-	Toxoid	-
20 Jun 80	637	-	Toxoid	-	Toxoid	-	-	Toxoid	-
24 Jun 80	641	Toxoid	-	-	-	Toxoid	-	Toxoid	-
1 Jul 80	648	-	Toxoid	Toxoid	-	-	-	Toxoid	-
8 Jul 80	655	-	-	-	-	-	-	Toxoid	-
8 Nov 80	778	-	Toxoid	-	-	-	-	Toxoid	-

IMMUNIZATION SCHEDULE AFTER ARRIVAL IN MINNESOTA

13 Jan 81	844	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-
14 Jan 81	845	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-
2 Mar 81	892	Toxoid	Toxoid	-	-	Toxoid	Toxoid	Toxoid	-
26 Apr 81	947	Toxoid	Toxoid	-	Toxoid	Toxoid	Toxoid	Toxoid	-

Table II

IMMUNIZATION SCHEDULE FOR "ABE"
HEPTAVALENT BOTULINUM ANTITOXIN

Date	Day	T O X I N T Y P E							COMMENT
		A	B	C	D	E	F	G	
10 Sep 81	1	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	5 ml, Adsorbed Penta.
23 Oct 81	43								5 ml Botulinum Toxoid
16 Nov 81	67								10 ml Botulinum Toxoid
18 Nov 81	69	-	-	-	-	Toxoid	-	-	5 ml, Adsorbed Mono.
18 Dec 81	99	-	-	-	-	-	Toxoid	-	2 ml, 50:50 Freund's
22 Dec 81	103	-	-	-	-	-	-	Toxoid	2 ml, 50:50 Freund's
08 Jan 82	120	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	F-1	Toxoid	4 ml, Adsorbed Penta.;
15 Jan 82	127	-	-	-	-	-	Toxoid	Toxoid	2 ml each F-1 & G
									1 ml each
22 Jan 82	134	-	-	-	-	-	F-1	Toxoid	1 ml each, IM
26 Jan 82	133	Toxin	Toxoid	-	-	-	Toxoid	Toxoid	A, 1 ml, SQ
									B, 5 ml, IM
16 Jun 82	279	Toxin	Toxin	Toxin	-	Toxin	Toxin	Toxin	F & G, 1 ml ea, IM
14 Jul 82	307	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	SQ
29 Jul 82	322	Toxoid*	Toxoid*	Toxoid	Toxoid	Toxoid*	-	Toxoid	SQ
									*2 X normal dose
									10 ml total
IMMUNIZATION SCHEDULE AFTER ARRIVAL IN MINNESOTA									
09 Jan 83	486	Toxoid	Toxoid	Toxoid	Toxoid	Toxoid	-	-	5 ml total
17 Apr 83	584	Toxin	Toxin	-	-	Toxin	-	-	1 ml each
21 Aug 83	710	Toxin	Toxin	Toxin	-	Toxin	Toxin	Toxin	6.5 ml total

FIRST FLIGHT

Bleed No.	Date	Blood Volume	Plasma Volume	Total Protein (mg/ml)	%IgG	IgG (mg/ml)	IgG(T) (mg/ml)
1	12/07/80	13.0	6.8	54.2	26.0	14.1	7.7
2	12/09/80	13.0	6.97	54.2	25.3	13.7	7.4
3	12/11/80	13.0	6.35	50.6	25.1	13.2	6.5
4	12/13/80	14.0	7.15	47.0	24.5	11.5	5.0
5	12/20/80	14.0	6.8	49.2	20.7	10.2	5.0
6	12/22/80	14.0	6.3	41.9	23.2	9.7	3.8
7	12/24/80	14.0	6.5	42.8	22.2	9.5	3.5
8	12/26/80	13.0	6.1	42.0	21.7	9.1	3.2
Immunized January 13, 1981 January 14, 1981				Botulinal Toxin Types A,B,C,D,E,F,G " " A,B,C,D,E,F,G			
9	01/21/81	12.0	7.43	60.0	27.0	16.2	6.8
10	01/23/81	12.0	7.36	55.9	29.0	16.2	7.0
11	01/25/81	12.0	6.87	50.7	29.8	15.1	6.6
12	01/27/81	12.0	6.68	46.2	27.7	12.8	5.2
13	02/03/81	12.0	6.3	51.3	29.6	15.2	5.3
14	02/05/81	12.0	6.35	46.3	30.2	14.0	4.8
15	02/07/81	12.0	6.70	42.5	28.6	12.2	4.0
16	02/09/81	12.0	5.35	41.0	28.8	11.8	3.5
Immunized March 2, 1981				Botulinal Toxin Types A,B,E,F,G			
17	03/09/81	12.0	8.37	65.0	22.2	14.4	7.5
18	03/16/81	12.0	7.22	61.9	27.9	17.3	11.7
19	03/18/81	12.0	7.20	60.7	26.0	15.8	7.0
20	03/20/81	12.0	7.00	54.0	27.0	14.6	9.7
21	03/22/81	12.0	6.40	53.4	25.5	13.6	9.0
22	03/29/81	12.0	7.50	50.7	22.1	11.2	6.3
23	03/31/81	12.0	6.40	51.2	22.1	11.3	5.9
24	04/02/81	12.0	6.50	46.4	20.0	9.3	4.9
25	04/04/81	12.5	7.05	47.1	19.1	9.0	5.0
Immunized April 26, 1981				Botulinal Toxin Types A,B,D,E,F,G			
26	05/05/81	12.0	7.6	60.8	28.9	17.6	7.4
27	05/07/81	12.0	7.5	55.8	28.7	16.0	5.6
28	05/09/81	12.0	7.0	51.3	25.7	13.2	5.9
29	05/11/81	12.0	7.0	52.4	27.3	14.3	6.2
30	05/18/81	12.0	6.5	51.2	23.8	12.2	4.7
31	05/20/81	12.0	6.0	50.0	23.8	11.9	4.4
32	05/22/81	12.0	7.3	43.1	22.5	9.7	3.3
33	05/24/81	12.0	7.4	44.2	21.0	9.3	3.4

Table III
Pg. 2 of 4

FIRST FLIGHT

<u>Bleed No.</u>	<u>Date</u>	<u>Blood Volume</u>	<u>Plasma Volume</u>	<u>Total Protein (mg/ml)</u>	<u>%IgG</u>	<u>IgG (mg/ml)</u>	<u>IgG(T) (mg/ml)</u>
Immunized October 11, 1981		Botulinal Toxin Types A,B,C,D,E,F,G					
34	10/18/81	12.0	6.75	60.0	30.3	18.2	5.25
35	10/21/81	12.0	7.10	55.2	32.8	18.1	5.23
36	10/24/81	12.0	6.65	53.8	32.2	17.3	4.75
37	10/27/81	13.0	7.3	49.3	31.2	15.4	3.84
38	10/30/81	12.0	6.5	53.1	28.6	15.2	3.94
39	11/02/81	12.0	5.95	47.4	26.4	12.5	3.01
40	11/05/81	12.0	6.10	46.1	27.1	12.5	2.84
41	11/08/81	12.0	6.00	46.6	25.3	11.8	2.76
Immunized December 13, 1981		Botulinal Toxin Types A,B,C,D,E,F,G					
42	12/22/81	12.0	7.15	60.4	32.0	18.9	3.2
43	12/24/81	12.0	6.6	57.9	31.5	18.5	3.4
44	12/26/81	12.0	7.0	52.0	30.0	16.4	2.9
45	12/28/81	12.0	7.2	48.3	28.5	14.5	2.7
46	01/04/82	12.0	6.7	47.4	28.5	13.5	2.2
47	01/06/82	12.0	6.3	49.8	27.1	13.5	2.2
48	01/08/82	12.0	6.4	46.2	26.6	12.3	2.0
49	01/10/82	12.0	4.95	46.8	25.6	12.0	1.8
Immunized February 8, 1982		Botulinal Toxin Types A,B,C,D,E,F,G					
50	02/17/82	12.0	7.1	62.1	30.1	18.7	6.5
51	02/19/82	12.0	6.0	59.5	28.7	17.1	5.8
52	02/21/82	12.0	6.45	56.3	26.8	15.1	5.0
53	02/23/82	12.0	6.1	50.0	26.6	13.3	4.2
54	03/02/82	12.0	6.7	50.3	26.4	13.3	4.0
55	03/04/82	12.0	6.6	47.8	25.3	12.1	3.7
56	03/06/82	12.0	6.6	46.7	23.6	11.0	3.2
57	03/08/82	12.0	6.3	43.3	21.0	9.1	2.5
Immunized May 2, 1982		Botulinal Toxin Types A,B,C,D,E,F,G					
58	05/11/82	12.0	6.8	65.5	26.6	17.4	6.5
59	05/13/82	12.0	6.9	60.5	28.8	17.4	6.1
60	05/15/82	12.0	6.5	53.5	28.6	15.3	5.2
61	05/17/82	12.0	6.9	54.3	26.5	14.4	4.9
62	05/24/82	12.0	6.82	51.9	27.0	14.0	4.4
63	05/26/82	12.0	6.28	52.9	25.9	13.7	4.4
64	05/28/82	12.0	7.0	48.7	27.1	13.2	4.0
65	05/30/82	12.0	5.9	44.2	27.1	12.0	3.6

Table III
Pg. 3 of 4

FIRST FLIGHT

<u>Bleed No.</u>	<u>Date</u>	<u>Blood Volume</u>	<u>Plasma Volume</u>	<u>Total Protein (mg/ml)</u>	<u>%IgG</u>	<u>IgG (mg/ml)</u>	<u>IgG(T) (mg/ml)</u>
Immunized June 20, 1982		Botulinal Toxin Types A,B,C,D,E,F,G					
66	06/29/82	12.0	7.5	58.6	22.5	13.2	4.5
67	07/01/82	12.0	6.85	59.9	21.4	12.8	4.5
68	07/03/82	12.0	6.8	56.5	24.2	13.7	4.2
69	07/05/82	12.0	6.6	57.6	16.7	9.6	3.9
70	07/12/82	12.0	6.8	50.7	18.1	9.2	3.7
71	07/14/82	12.0	6.75	47.4	21.1	10.0	3.4
72	07/16/82	12.0	6.5	47.5	20.2	9.6	3.4
73	07/18/82	12.0	5.8	45.9	17.2	7.9	3.0
Immunized August 1, 1982		Botulinal Types A,B,C,E,F,G					
74	08/10/82	12.0	6.6	65.2	20.6	13.4	8.8
75	08/12/82	12.0	6.8	60.6	26.2	15.9	8.1
76	08/14/82	12.0	7.1	57.2	24.5	14.0	5.8
77	08/16/82	12.0	6.9	47.0	22.1	10.4	5.4
78	08/23/82	12.0	6.5	51.8	18.5	9.6	5.5
79	08/25/88	12.0	6.6	50.6	16.8	8.5	4.9
80	08/27/82	12.0	6.5	44.0	16.0	7.1	3.7
81	08/29/82	12.0	6.5	41.8	17.0	7.1	3.3
Immunized September 26, 1982		Botulinal Toxin Types A,B,C,E,G					
82	10/05/82	13.0	7.15	65.2	21.3	13.9	6.2
83	10/07/82	11.5	5.1	59.2	20.6	12.2	5.4
84	10/09/82	12.0	6.5	54.2	21.2	11.5	5.0
85	10/11/82	12.0	7.4	53.0	21.7	11.5	4.8
86	10/18/82	12.0	6.7	54.4	22.4	12.2	4.6
87	10/20/82	12.0	7.1	49.6	20.8	10.3	4.0
88	10/22/82	12.0	5.25	44.8	19.6	8.8	3.6
89	10/24/82	14.0	7.85	45.5	18.0	8.2	3.6
Immunized November 7, 1982		Botulinal Toxin Types A,B,E,G					
90	11/15/82	12.0	7.8	58.0	23.1	13.4	2.9
91	11/17/82	12.0	7.4	57.2	23.4	13.4	3.3
92	11/19/82	12.0	7.35	52.0	24.0	12.5	2.6
93	11/21/82	12.0	7.22	50.0	26.0	13.0	2.8
94	11/28/82	12.0	7.15	54.2	25.7	13.9	2.8
95	11/30/82	12.0	7.6	53.8	24.2	13.0	2.7
96	12/02/82	12.0	6.7	48.8	21.5	10.5	2.2
97	12/04/82	12.0	6.7	43.2	20.4	8.8	2.0

Table III
Pg. 4 of 4

FIRST FLIGHT

<u>Bleed No.</u>	<u>Date</u>	<u>Blood Volume</u>	<u>Plasma Volume</u>	<u>Total Protein (mg/ml)</u>	<u>%IgG</u>	<u>IgG (mg/ml)</u>	<u>IgG(T) (mg/ml)</u>
Immunized January 9, 1983			Botulinal Toxin Types A,B,C,D,E				
98	01/18/83	12.0	7.2	61.5	22.4	13.8	5.9
99	01/20/83	12.0	7.1	54.0	18.0	9.7	4.2
100	01/22/83	12.0	7.2	53.7	19.0	10.2	4.0
101	01/24/83	12.0	6.7	49.2	16.9	8.3	3.4
102	01/31/83	12.0	6.4	52.5	14.9	7.8	3.6
103	02/02/83	12.0	7.1	48.0	15.4	7.4	3.0
104	02/04/83	12.0	6.9	43.8	12.1	5.3	2.7
105	02/06/83	12.0	7.6	42.9	15.4	6.6	2.5
Immunized April 17, 1983			Botulinal Toxin Types A,B,E				
106	04/26/83	12.0	6.4	61.5	22.8	14.0	5.4
107	04/28/83	12.0	6.1	55.1	24.7	13.6	4.9
108	04/30/83	12.0	6.6	59.3	21.4	12.7	4.3
109	05/02/83	12.0	6.8	52.9	21.7	11.5	3.7
110	05/09/83	12.0	6.7	No sample			
111	05/11/83	12.0	6.15	No sample			
112	05/13/83	12.0	7.0	44.2	20.8	9.2	3.0
113	05/15/83	12.0	6.3	42.7	19.9	8.5	2.6
Immunized August 21, 1983			Botulinal Toxin Types A,B,C,E,F,G				
Pre-Imm	08/21/83			68.5			
114	08/30/83	12.0	7.5	62.1	13.4	21.6	6.5
115	09/01/83	12.0	6.6	55.0	12.4	22.5	6.0
116	09/03/83	12.0	5.45	50.6	15.2	30.0	5.5
117	09/05/83	12.0	6.5	49.9	15.2	30.5	5.0
118	09/12/83	12.0	6.2	53.6	12.5	23.3	5.5
119	09/14/88	12.0	6.25	47.1	11.2	23.8	4.5
120	09/16/83	12.0	NR*	43.5	8.8	20.2	4.0
121	09/18/83	12.0	6.95	42.8	7.0	16.4	3.5

*NR = Not Recorded

Table IV

ABE

Total Protein, IgG, IgG(T) Levels

Immunized January 9, 1983

Botulinal Toxin Types A,B,C,D,E,

Bleed No.	Date	Blood Volume	Plasma Volume	Total Protein (mg/ml)	%IgG	IgG (mg/ml)	IgG (T) (mg/ml)
1	1/18/83	12.0	6.5	61.1	26.2	16.0	4.4
2	1/20/83	12.0	6.1	55.2	22.8	12.8	3.9
3	1/22/83	12.0	6.4	50.3	22.3	11.2	3.3
4	1/24/83	12.0	5.6	47.5	24.8	11.8	3.1
5	1/31/83	12.0	7.0	51.5	27.0	13.9	3.1
6	2/02/83	12.0	7.0	44.7	24.4	10.9	2.7
7	2/04/83	12.0	6.8	42.7	22.7	9.7	2.7
8	2/06/83	12.0	6.3	41.7	23.0	9.6	2.8

Immunized April 17, 1983

Botulinal Toxin Types A,B,E

9	4/26/83	12.0	7.3	59.8	30.8	18.4	4.4
10	4/28/83	12.2	7.3	57.9	31.8	18.4	3.7
11	4/30/83	12.0	6.4	48.5	29.7	14.4	3.2
12	5/02/83	12.0	6.8	48.2	29.0	14.0	3.0
13	5/09/83	12.0	6.05	64.6	29.1	18.8	3.7
14	5/11/83	12.0	6.1	44.5	29.4	13.1	2.4
15	5/13/83	12.0	5.9	41.6	26.0	10.8	2.0
16	5/15/83	12.0	5.5	39.5	25.3	10.0	2.6

Immunized August 21, 1983

Botulinal Toxin Types A,B,C,E,F,G

17	8/30/83	12.0	7.6	64.3	25.0		4.5
18	9/01/83	12.0	7.6	57.7	25.0		4.5
19	9/03/83	12.0	7.3	53.5	25.0		4.5
20	9/05/83	12.0	6.7	50.7	16.4		4.0
21	9/12/83	12.0	6.45	54.6	23.8		3.5
22	9/14/83	12.0	6.0	45.9	15.2		3.0
23	9/16/83	12.0	5.95	43.8	15.2		2.5
24	9/18/83	12.0	6.25	42.9	15.2		2.5

Table IV

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Acknowledgement

We acknowledge the contributions of Karen S. Alsop, Ted Taylor, Pamela J. Vaughan, and Christopher P. Drayton in the preparation of this report.

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